# EXPLORATION OF BODY WEIGHT REGULATION BY POLYUNSATURATED FATTY ACIDS IN RAT: POTENTIAL ASSOCIATION WITH HYPOTHALAMIC NEUROGENESIS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

Submitted by

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## DECLARATION

Apart from help and advice acknowledged, this thesis represents the unaided work of the author

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## Acknowledgements

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## Abstract

Of the environmental factors which influence body weight, nutrients have the most impact. Saturated fatty acids (SFAs) are a macronutrient, which induce obesity, characterised by metabolic dysfunction and altered feeding behaviour. Currently, there are no effective pharmaceutical treatments for obesity. Nutraceutical intervention, including consumption of polyunsaturated fatty acids (PUFAs), represents a promising alternative. PUFAs oppose the effects of SFAs, attenuating weight gain by enhancing satiety. However, in studies using human and rodent models findings are difficult to interpret, due to variation in protocols. The primary aim of this project was to create a rational model of chronic PUFA consumption.

Obesity is underpinned by alterations in hypothalamic neuronal plasticity including impairment of neurogenesis. These changes occur in response to nutrients or by regulation of appetite-related hormones. In contrast, dietary restriction stimulates neurogenesis, and evidence has suggested that PUFAs enhance hypothalamic neurogenesis. The secondary aim of this project was to pilot methods for stimulating and observing neurogenesis in hypothalamus of rat. These two lines of enquiry were pursued to begin addressing the complex research question of whether PUFAs exert beneficial effects on body weight by stimulating hypothalamic neurogenesis.

Wistar rats fed commercially formulated diets equally enriched with high concentrations of SFAs and PUFAs, from lard and fish oil, respectively, for two months showed no difference in energy intake or body weight. Both diets induced leptin and insulin resistance, but PUFAs reduced triglyceride concentrations. Hence, PUFAs improved lipid metabolism independently of induced obese phenotype. The meal pattern signatures associated with each diet were also similar; however, PUFA-fed animals demonstrated enhanced diurnal satiety. Closer examination of the diet compositions revealed the overlap of results was likely due to the presence of SFAs in the PUFA diet. This led to reformulation, using fatty acids of greater purity. The chosen sources were coconut oil (SFAs), and a commercial preparation of omega-3 PUFAs. A shorter-term, preliminary investigation involving three weeks' dietary exposure was conducted. Energy intake was again similar between SFA- and omega-3-fed animals but weight gain was attenuated and adiposity reduced by omega-3 feeding. However, the enhanced satiety previously observed was not borne out. A rise in concentration of brain-derived neurotrophic factor, known to be associated with beneficial effects of PUFA intake, in the final study week suggests that studies of longer duration may be required to fully assess the effects of dietary PUFAs.

Concurrent pilot work showed that hypothalamic cell proliferation could be stimulated in response to simple enrichment (play tube) introduced to the cage. However, a full study failed to repeat these findings, supporting the notion that neurogenesis is subject to many influences, including age, species, strain and stress, the degrees of influence of which would have to be determined in a series of systematically controlled studies. Failure to stimulate cell proliferation in PUFA-fed rats suggested further that change in dietary fatty acid composition is not a powerful enough intervention to stimulate neurogenesis, when used alone in Wistar rats.

In conclusion, application of appropriate controls for dietary energy content and composition show that benefits to body weight metabolism of long-term consumption of diets highly enriched with PUFAs, and, in particular, omega-3 fatty acids can be successfully modelled in rat. However, further work is required to determine the precise timeline of their emergence and underlying mechanisms.

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## List of Abbreviations

**3V:** third ventricle

**4-AAP**: 4-aminoantipyrine

**5-HT**: serotonin

**A4.74:** fast fibre type

**A4.840:** slow fibre type

**ABTS:** 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

**ACh:** acetylcholine

**AgrP:** agouti-related peptide

**AMP:** adenosine monophosphate

**ANOVA:** analysis of variance

**ARC:** arcuate nucleus

**as:** antisense

**ATP:** Adenosine-5'-triphosphate

**AUC:** area under the curve

**BAT:** brown adipose tissue

**BBB:** blood-brain barrier

**BDNF:** brain-derived neurotrophic factor

**bHLH**: basic helix-loop-helix

**BMI:** body mass index

**BMR:** basal metabolic rate

**BrdU:** bromo-5-deoxyuridine

**BSA**: bovine serum albumin

**BSS:** behavioural satiety sequence

**CA2:** region in the hippocampus

**CART**: cocaine and amphetamine-regulated transcript

**CCK**: cholecystokinin

**cDNA**: complementary single-stranded DNA

**CNS**: central nervous system

**CNTF:** ciliary neurotrophic factor

**COOH:** carboxyl

**COPD:** chronic obstructive pulmonary disease

**CORT:** corticosterone

**CSF:** cerebrospinal fluid

**CT:** threshold cycle

**DAP:** dihydroxyacetone phosphate

**DAPI**: 4',6-diamidino-2-phenylindole

**DCX:** doublecortin

**df:** degrees of freedom

**DF:** dilution factor

**DG:** dentate gyrus

**DHA:** docosahexaenoic acid

**DIO**: diet-induced obesity

**DMH**: dorsomedial hypothalamus

**DNA:** Deoxyribonucleic acid

**DR**: resistancy to diet-induced obesity

**EdU**: 5-ethynyl-2′-deoxyuridine

**EDL**: extensor digitorum longus

**EGF**: Epidermal growth factor

**EIA:** enzyme immunoassay

**ELISA:** enzyme-linked immunosorbent assay

**EPA:** eicosapentaenoic acid

**ESPA:** sodium *N*-ethyl-*N*-(3-sulfopropyl) *m*-anisidine

**FA:** fatty acid

**FAM**: 6-carboxyfluorescein

**FAME:** fatty acid methyl ester

**FFAs**: free fatty acids

**FFM:** fat free mass

**fMRI**: functional magnetic resonance imaging

**Fig**: figure

**FRET**: fluorescence resonance energy transfer

**g:** grams

**GAD:** glutamic acid decarboxylase

**GAPDH:** glyceraldehyde-3-phosphate

**GC:** guanine-cytosine

**GDP**: Guanosine diphosphate

**GK:** glycerol kinase

**GL:** granular layer

**GLP-1**: glucagon-like peptide-1

**Glut2:** Glucose transporter 2

**GPO:** glycerol phosphate oxidase

**GPRs:** G-protein-coupled receptors

**GRH**: growth hormone-releasing factors

**HCl**: hydrochloric acid

**HFCS**: high-fructose corn syrup

**HFD:** high-fat diet

**HPA:** hypothalamic-pituitary-adrenal axis

**$H_2O$**: water

**$H_2O_2$:** hydrogen peroxidise

**HSCIC**: The Health and Social Care Information

**ICC:** immunocytochemistry

**i.c.v.:** intracerebroventricular

**ID:** identification number

**IGF**: insulin-like growth factor

**IgG**: immunoglobulin

**IHC:** immunohistochemistry

**IKKβ:** IκB kinase β

**IMI:** intermeal interval

**i.p.:** intraperitoneal

**i.v.:** intravenous

**JNK:** c-Jun N-terminal kinases

**$K_{ATP}$:** ATP sensitive potassium channel

**LBM:** lean body mass

**LE:** Long-Evans

**LFD:** low-fat diet

**LHA:** lateral hypothalamic area

**LPL:** lipoprotein lipase enzyme

**LV**: lateral ventricle

**m:** murine

**MCH:** melanin-concentrating hormone

**ME:** median eminence

**mg**: milligram

**min:** minutes

**ml:** millilitre

**mM**: millimolar

**MPs:** meal patterns

**mRNA**: messenger RNA

**MUFA:** monounsaturated fatty acids

***n:*** number

**nm**: nanometre

***n*-3**: omega 3 PUFAs

**NF-κB**: nuclear factor –κB

**NGF:** nerve growth factor

**NHS:** National Health Service

**NMDA:** *N*-methyl-D-aspartate

**NPCs:** neural progenitor cells

**NPY:** neuropeptide Y

**NSCs:** neural stem cells

**NTS:** nucleus of the solitary tract

**OD:** optical density

**P**: probe

**PB**: phosphate buffer

**PBS:** phosphate buffered solution

**PCNA:** proliferating cell nuclear antigen

**PCR:** polymerase chain reaction

**POD:** peroxidase

**POMC**: pro-opiomelanocortin

**PPARs:** peroxisome proliferator-activated receptors

**PUFA:** polyunsaturated fatty acid

**PVN:** paraventricular nuclei

**PYY:** peptide YY

**qPCR:** Real-time PCR

**RAR:** retinoic acid receptors

**RIA:** radioimmunoassay

**ROX:** carboxy-X-rhodamine

**RNA:** ribonucleic acid

**RNAPolII**: RNA polymerase II

**rpm:** revolutions per minute

**RXR:** retinoid X receptor

**s:** sense

**S:** s phase

**SCN:** suprachiasmatic nucleus

**SD**: standard deviation

**sec:** seconds

**SEM**: standard error of the mean

**SFA:** saturated fatty acid

**SGL:** subgranular layer

**SGZ:** subgranular zone

**SVZ:** subventricular zone

**$T_a$(°C):** annealing temperature

**TAMRA**: 6 carboxytetrametheyrhodamine

**TBE**: tris-borate-EDTA

**TBF:** total body fat

**TD:** termination day

**TG**: triglyceride

**TMB:** 3,3',5,5'-tetramethylbenzidine

**TNF-α**: tumor necrosis factor-alpha

**TOBEC**: total-body electrical conductivity

**TrkB**: Tyrosine-related kinase B

**UCP:** uncoupling protein

**µl:** microlitre

**U/ml:** units per millilitre

**U/µl**: units per microlitre

**UV**: ultra violet

**VMH:** ventromedial hypothalamus

**v/v:** volume to volume

**WAT:** white adipose tissue

**WHO:** world health organisation

**wt/wt:** weight to weight

**w/v:** weight to volume

**ZDF**: Zucker fatty diabetic rat

**%**: percentage

**α-MSH**: alpha-melanocyte- stimulating hormone

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# CHAPTER 1

## GENERAL INTRODUCTION

# General Introduction

## 1.1 Energy Homeostasis

### 1.1.1 Regulation of Appetite & Body Weight

Energy homeostasis, which is essential for survival, is maintained by a balance between energy intake and energy expenditure. This concept is known as the energy balance equation (see Figure 1.1; Harvey & Withers, 2008).

### 1.1.2 Energy Balance Equation

Energy is readily available from the foods we consume; this particular source is known as 'Energy In'. Although food items contain a number of nutrient sources, energy is provided primarily by sources of protein, carbohydrate, and fat (Schoeller, 2009).

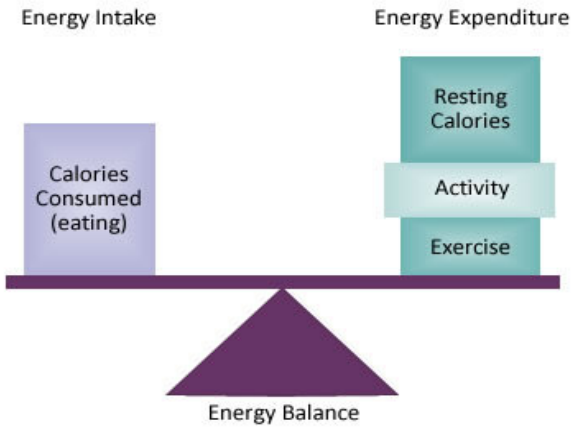



**Figure 1.1. Example of a normal energy balance.** Energy homeostasis is maintained by a balance between energy intake and energy expenditure. Source: Lowrie, 2008. *Human Biology*. Heinemann Publishing.

### 1.1.3 Controlling Energy Intake

'Energy In' is exclusively regulated by the amount of energy consumed. This occurs mainly through decreasing portion size and/or regulating the quantity of high fat and refined sugar items as well as 'calorie counting'. Energy in is also influenced by the opposing sensations of hunger and satiety (Schoeller, 2009). Hunger is a physiological state controlled by internal factors involving the nervous and endocrine systems; some of which are described in more detail in the following sections of this

thesis. Physiological factors that may lead to decreased energy intake include increased circulating concentrations of peptides, proteins and hormones, as well as increased temperature, stress, sensory mechanisms and conditioned responses whereby one trains oneself to eat less. However, the peptides, proteins and hormones that lead to increased food intake have not been completely characterised. Furthermore, there are also sensory mechanisms linked to smell and palatability of a food item that may influence food intake (Kalra & Kalra, 1990). Regulation to control 'Energy In' is complex and involves the interaction of many signals.

### 1.1.4 Controlling Energy Expenditure

In comparison, energy expenditure is represented by 'Energy Out' and has three constituents: 1) an individual's basal metabolic rate (BMR), 2) the energy used for physical activity, and 3) the thermic effect of food (Prentice & Jebb, 2004). The majority of an individual's daily energy expenditure (approximately 70%) is spent on the basal metabolic rate. BMR represents the energy used to carry out basic metabolic processes, within the body, such as breathing, regulating body temperature and maintaining a heartbeat (Zakeri *et al.,* 2008). The amount of energy required for these processes varies depending on factors such as age, growth, body composition (Fulton *et al.,* 2009), stress, surrounding temperature and fasting. Physical activity amounts to approximately 25% of the body's energy output, which varies dependent on type and duration of the activity. This is also affected by factors such as gender, age and weight of the person. Finally, the thermic effect of food refers to the energy necessary for the digestion of food consumed as food items need to be broken down into more usable structures within the body for later use as an energy source (Simopoulos, 1987; Rolland-Cachera *et al.,* 1997; Hill *et al.,* 2003). This energy balance equation remains neutral in instances where the energy consumed is equal to the energy expended through exercise and metabolism (Harvey & Withers, 2008). The control mechanisms which regulate energy consumption are described in the following sections.

### 1.1.5 Forms of Energy Balance

Although energy intake and expenditure are often variable from day to day, they tend to remain equal over long periods of time in order to maintain a stable body weight. However, sometimes the mechanisms described above are altered due to external

factors and an imbalance may occur. This imbalance will either result in excess body fat accumulation, if there is a surplus of energy, or in wasting and cachexia, if there is an energy deficit. Both conditions are accompanied by changes in morphology and regulation of energy homeostasis, and very importantly constitute health threatening conditions. In the following sections, examples of both negative and positive energy balances are discussed; however, it is situations of positive energy balance which are the emphasis of this review.

### 1.1.5.1 Negative Energy Balance

Individuals suffering from an insufficient intake of energy and/or a lack of specific nutrients are thought to be malnourished. This is an example of a negative energy balance (see Figure 1.2). Negative energy balance is dependent on a number of factors including age, sex, height, weight and waist measurements (Parvanta *et al.,* 1994).

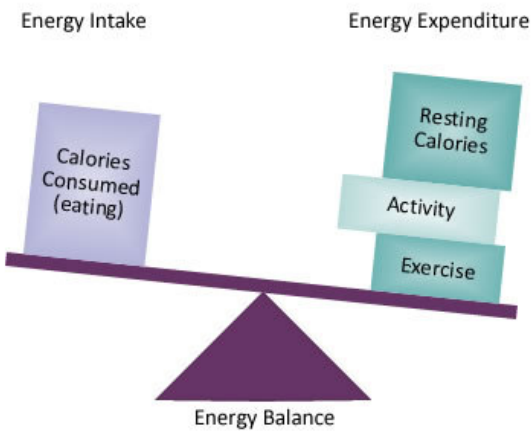



**Figure 1.2. Example of a negative energy balance.** When energy expenditure is greater than energy intake then a negative energy balance occurs. Source: Lowrie, 2008. *Human Biology.* Heinemann Publishing.

Examples of negative energy balance include anorexia, which is characterised by a significant weight loss as a result of excess energy restriction (Huse & Lucas, 1999), bulimic tendencies (Yanovski, 1999), and cachexia where an individual experiences weight loss, muscle wastage and loss of appetite (Lainscak *et al.,* 2007). However, negative energy balance can also occur in normal situations such as lactation. Many mammals use the energy reserves stored during pregnancy in

early lactation to support milk production but most mothers then go through a negative energy situation whereby energy consumption does not meet the demands of the milk production which often results in weight loss (Barbosa *et al.,* 1997; McGuire *et al.,* 2004).

### 1.1.5.2 Positive Energy Balance

Positive energy balance occurs when more energy is consumed than expended (see Figure 1.3). Increased consumption and subsequent weight gain can be adaptive in the short term, for example, in hibernation (Forbes, 2000) where excess fat stores allow the animal to survive throughout the winter, but is maladaptive in the longer term, as shown clearly by the metabolic dysregulation and co-morbidities associated with weight gain and ultimately obesity (Storlien *et al.,* 1991; Cruciani-Guglielmacci *et al.,* 2005; Schelbert, 2009).

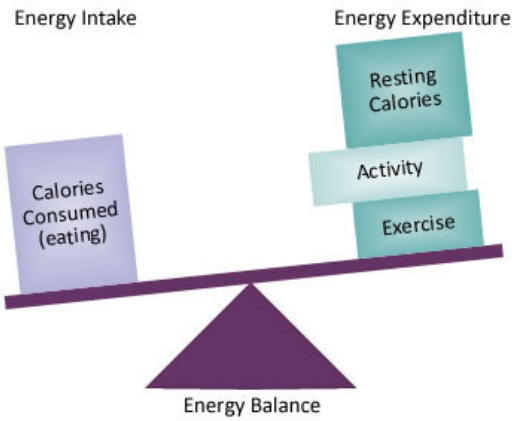



**Figure 1.3. Example of a positive energy balance.** When energy intake is greater than energy expenditure then a positive energy balance occurs. Source: Lowrie, 2008. *Human Biology.* Heinemann Publishing.

### 1.1.6 Regulation of Appetite: Homeostatic *vs.* Hedonistic Control

Energy consumption is controlled by both metabolic and non-metabolic influences. In humans, the non-metabolic, or non-homeostatic, influences include those of the environment, cognition, emotion and reward systems. The latter three components are involved in the hedonic control of appetite. These factors are co-ordinated by the limbic system which incorporates the ventral striatum, prefrontal cortex and amygdala regions of the brain, whereas, the homeostatic influences originate from

the hypothalamus (see Figure 1.4). The hedonistic control mechanisms can override the homeostatic influence (Berthoud, 2006; 2011).

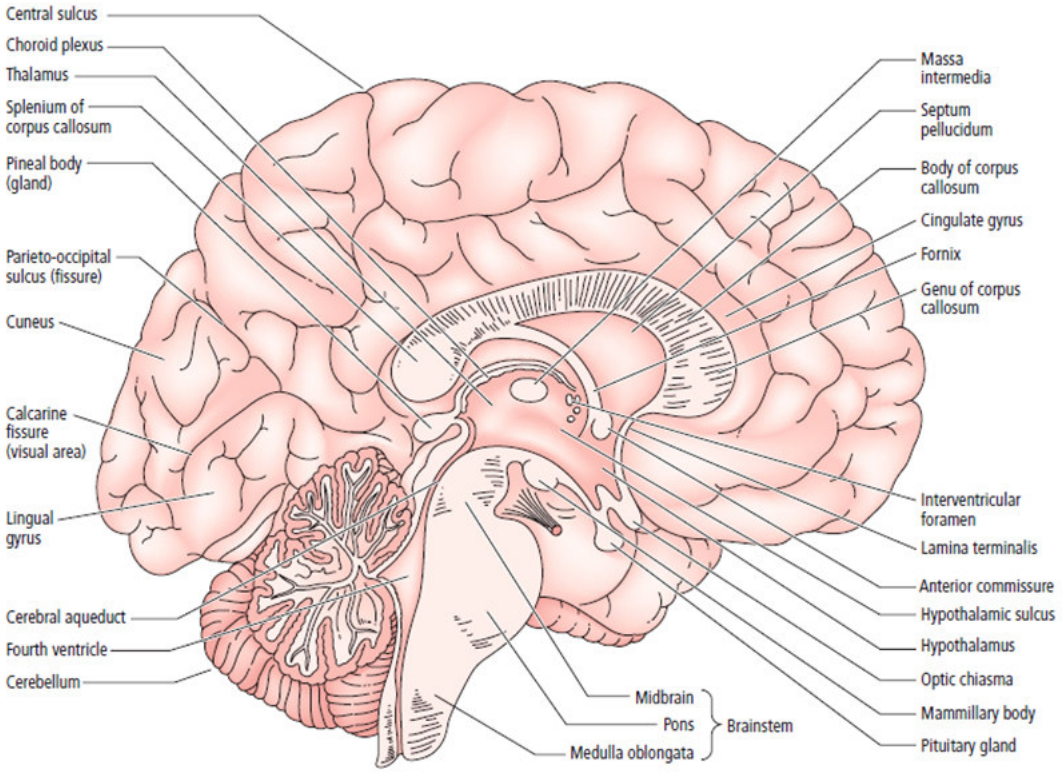



**Figure 1.4. Diagram of the medial view of a sagittal section of the brain.** The hypothalamus is positioned centrally in the brain and is involved in the regulation of energy homeostasis. Source: Fix, 2008. *Atlas of the Human Brain and Spinal Chord.* Jones and Bartlett Publishers.

### 1.1.6.1 Homeostatic Control of Appetite

The central nervous system (CNS) integrates inputs from all over the body to coordinate how the body acts. Within the CNS, the brain plays a specific role in monitoring the body's nutritional state through a complex system of neurons, which communicate via widespread circuitry (forebrain – hypothalamus – brainstem), as well as with peripheral organs (fat, liver, gut), through the substances they secrete (hormones, metabolites, neuropeptides) (see Figure 1.5). Furthermore, the hypothalamus, which is located adjacently to the third ventricle (3V), consists of neuronal populations, some of which are critically involved in the regulation of food intake (Schwartz *et al*., 2000). When food is eaten, it is broken down by the digestive system into nutrients, such as glucose (Levin *et al*., 2006) and fatty acids (FAs) (Migrenne *et al*., 2011) that circulate in the bloodstream and directly activate specific

neuronal populations in the hypothalamus. Also, these neuronal populations can be indirectly activated by changing levels of peripheral adiposity and appetite-regulating hormones, such as the adipokine, leptin (Chen *et al.,* 2008). This supports a primary role for the brain in the regulation of energy metabolism (Pella *et al.*, 2011; Södersten *et al.*, 2011), alongside growing knowledge of important peripheral drivers, such as lean body mass (Blundell *et al.,* 2012).

#### 1.1.6.2 Hedonic Control of Feeding

The process of eating is thought to initially start as a cognitive decision from the cortex despite the presence of energy stores. Therefore, even when satiated, the limbic (including the hippocampus) and cortex systems can override the hypothalamus to make an individual eat and often occurs in response to an external non-homeostatic factor such as the presence of readily available, palatable, energy high foods (see Figure 1.5; de Castro & Plunkett, 2002). The palatability of a food item is one of the major causes behind the process of energy intake. Palatable foods are thought to stimulate the brain's hedonistic reward pathways to override the homeostatic signals within the hypothalamus that would normally curb appetite (Bessesen, 2011). Despite the difficulties encountered by researchers when trying to establish the neurological and psychological control mechanisms behind the feeling of pleasure, when specifically applied to food intake, the emotion is often associated with the consumption of refined sugar and fatty food items (Kelley *et al.,* 2002). The nutrient sensory mechanisms behind this are discussed in the following sections. This process is thought to involve connective circuitry between the nucleus accumbens and the lateral hypothalamic area (LHA) (Mogenson *et al.,* 1980; Usuda *et al.,* 1998; Otake & Nakamura, 2000; Zahm, 2000). Several studies have shown that the activation of opioid receptors in regions of the nucleus accumbens stimulates high-fat feeding (Zhang & Kelley, 1997, 2000; Zhang *et al.,* 1998; Will *et al.,* 2003) whereas the blocking of glutamate receptors in the LHA via injections of agonists into the nucleus accumbens prevented food intake (Maldonado-Irizarry *et al.,* 1995). From a hedonic point of view, opioids are implicated in the consumption of highly palatable, energy dense foods, and craving for these foods is often considered as a form of opioid-influenced addiction (Nogueiras *et al.,* 2012).

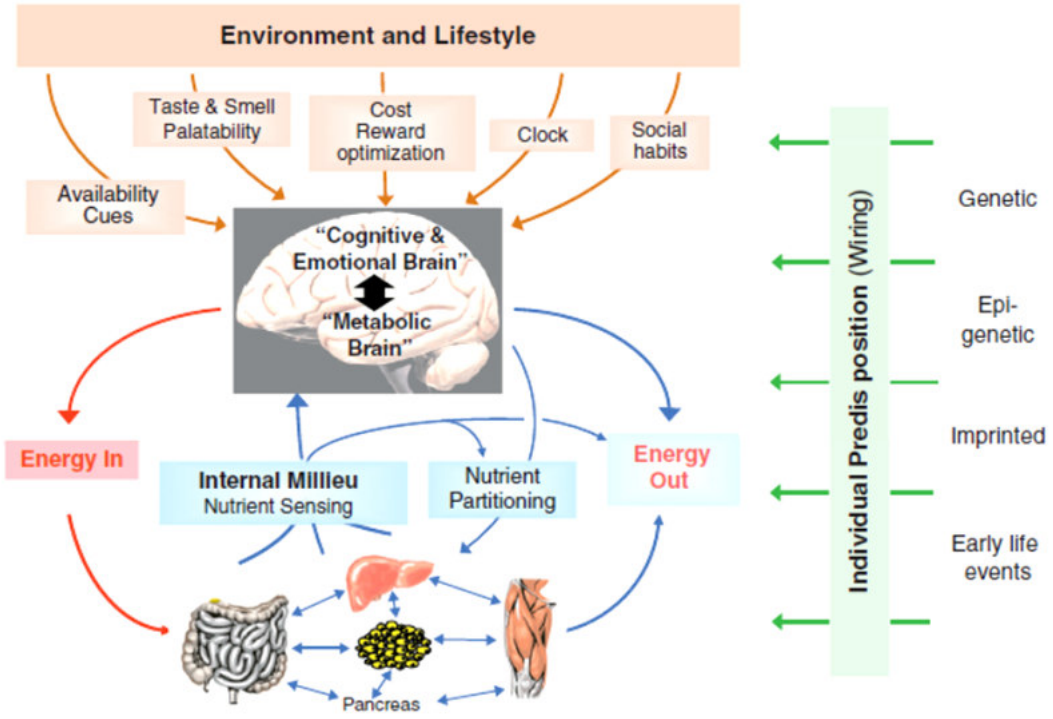



**Figure 1.5. Schematic diagram showing the major factors determining neural control of appetite and regulation of energy balance.** The brain monitors the internal milieu through a number of hormonal and neural nutrient sensing mechanisms and is under constant influence of the environment and lifestyle through the senses and mainly the cognitive and emotional brain. The two streams of information are integrated to generate adaptive behavioural (food intake) and autonomic/endocrine responses determining nutrient partitioning, energy expenditure and overall energy balance. All the peripheral and central signalling steps are subject to individual predisposition either through genetic, epigenetic or non-genetic early life imprinting mechanisms. Source: Berthoud, 2011. *Curr Opin Neurobiol* **21**(6):888-96.

### 1.1.7 Hypothalamic Neuronal Populations and Functions

Dietary nutrients can profoundly affect the brain's organisation and function, which can have extensive effects on physiology, metabolism and behaviour. Many advances have been made in defining the nature of CNS control over appetite and feeding behaviour. This research began with lesion studies in experimental animals, which identified "satiety" and "appetite" regions of the hypothalamus, and now includes defining the appetitive function of individual neuronal proteins, through their absence, in transgenic mice (for review see Coll *et al.,* 2007).

The suggested existence of "genes-to-behaviour" inter-relationships is evident from a combination of research findings in humans and rodent models. For instance, known gene defects contribute to the metabolic disturbance of obesity in

both species (Harvey & Withers, 2008), both of which also show similar disturbances in meal patterns (Castonguay *et al*., 1982; Berg *et al.,* 2009). An emerging area of particular interest suggests that dietary fatty acids, depending on their degree of saturation, exert differential effects on energy homeostasis in humans and rodent models (including meal patterns) (Cha & Jones, 1998; Lawton *et al.,* 2000). These effects appear to be underpinned by the degree of neuronal activation and proliferation in the brain (neurogenesis) (Wang *et al.,* 1999; Mattson *et al*., 2003; Kokoeva *et al.,* 2005).

### 1.1.8 Feeding Nuclei of the Hypothalamus

The specific hypothalamic nuclei which are associated with regulation of energy balance include the arcuate (ARC), ventromedial (VMH also known as the satiety centre), dorsomedial (DMH), paraventricular (PVN) nuclei and lateral hypothalamic area (LHA also known as the appetite centre) all of which surround the third ventricular cavity (3V) (see Figure 1.6). Specific neurons located in these nuclei produce chemical messengers, which stimulate or inhibit feeding behaviour. Due to the presence of a week blood–brain barrier (BBB) in the median eminence (ME), communication between the periphery and the brain is possible (Coll *et al.,* 2007). Some of the hypothalamic peptides produced by these nuclei are described in the following sections.

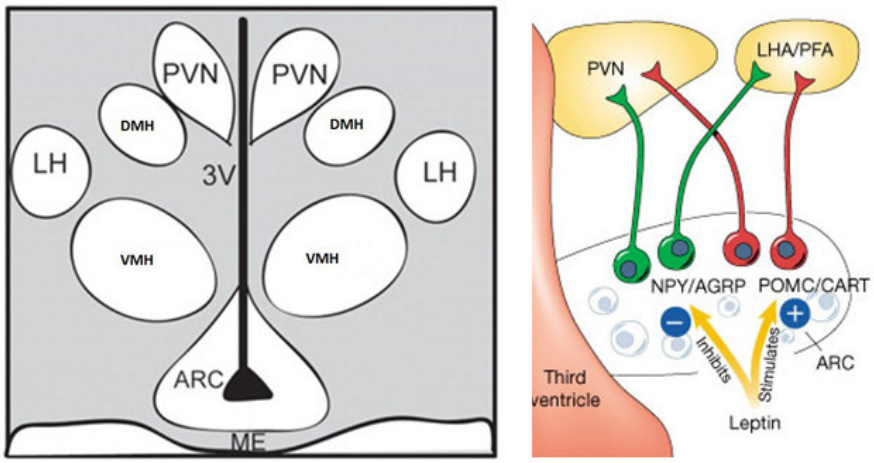



**Figure 1.6. Cross-sectional view of hypothalamic nuclei and key neuropeptides involved in feeding. Abbreviations:** AGRP= Agouti-related peptide; ARC=arcuate nucleus; CART= cocaine and amphetamine-regulated transcript; DMH=dorsomedial hypothalamic nucleus; LH/LHA= lateral hypothalamic nucleus; ME= median eminence; NPY= Neuropeptide Y; PFA= perifornical area; POMC= pro-opiomelanocortin; PVN=paraventricular nucleus; VMH=ventromedial hypothalamic nucleus; 3V=third ventricle. Source: Schwartz *et al*., 2000. *Nature* **404**(6778): 661-71.

### 1.1.9 Appetite-Related Signals in the Hypothalamus and CNS

Several hypothalamic peptides are essential to the control of homeostatic influenced energy balance (Elmquist, 2001). There is an extensive regulatory system to control energy homeostasis. Neurons present in the lateral and mediobasal hypothalamus control the autonomic, behavioural and endocrine systems responsible for energy intake and expenditure. There are many metabolic pathways in the hypothalamus: the PVN provides a link to the autonomic outflow through the medulla and spinal cord, as well as the endocrine axis. Other groups of neurons in the LHA connect the autonomic systems in the brainstem and neurons within the ARC are able to detect the presence of circulating factors and in doing so can then regulate the metabolic state of an individual (Berthoud, 2006). Neurons present in the hypothalamus produce mediators that have orexigenic or anorexigenic purpose, which are secreted within the hypothalamus, but are also projected to other regions of the brain, also involved in the control of feeding, such as the brainstem. Numerous peptides which stimulate or inhibit food intake have been discovered within the hypothalamus (Meister, 2007), some of which are described in the following sections.

#### 1.1.9.1 Arcuate Nucleus

The ARC, situated around the base of the 3V, lies immediately above the median eminence (Figure 1.6). The ARC is a collection of neuronal cell bodies and is subdivided into several functional categories. For example, neuropeptide Y (NPY) and agouti-related peptide (AgrP), both potent stimulators of food intake, are colocalised in a population of neurons in the ARC (Broberger *et al.,* 1997; Kalra *et al.,* 1999; Kamiji & Inui, 2007), while pro-opiomelanocortin (POMC; the precursor of alpha-melanocyte- stimulating hormone (α-MSH)) and cocaine- and amphetamine-regulated transcript (CART), which induce an anorexic response, are colocalised in an adjacent subset of ARC neurons (Elias *et al.,* 2001; Berthoud, 2006; Meister, 2007). These two populations interact with each other (see Figure 1.5). The ARC also has extensive connections with the PVN, DMH, VMH and LHA. The endocannabinoid neurotransmitters, which are synthesised in the ARC, are also known to regulate appetite (Matias & Di Marzo, 2007; Di Marzo *et al.,* 2009; Richard *et al.,* 2009). Homeostatic regulation is mediated in the ARC, PVN and through the nucleus of the solitary tract in the brainstem, and the hedonic regulation

is mediated in part through effects in the nucleus accumbens, hypothalamus and brainstem (Volkow *et al.,* 2011). Studies have shown that administration of endocannabinoid antagonists reduces energy intake and adiposity, and increases leptin sensitivity in rodent models (Ravinet Trillou *et al*., 2004; Di Marzo *et al.,* 2009).

#### 1.1.9.2 Paraventricular Nucleus

The PVN lies at the top of 3V and is an integrating centre, on which converge many neural pathways that influence energy homeostasis, and is abundantly supplied by axons projecting from the ARC, including NPY/AgrP and POMC/CART neurons and from the orexin neurons of the LHA (see Figure 1.6). The PVN is rich in terminals containing numerous appetite-modifying neurotransmitters, including NPY, α-MSH, serotonin (5-HT), galanin, noradrenaline and the opioid peptides, and the PVN is particularly sensitive to these neurotransmitters' effects on feeding and energy expenditures (Williams *et al.,* 2001). Noradrenaline is secreted into the hypothalamus from the brainstem *via* specific fibres that are known as noradrenergic fibres. Lesions to these fibres results in over eating and obesity, whereas the administration of noradrenaline into the PVN of the hypothalamus reduces eating by affecting satiety (Schwartz *et al.,* 2000). Serotonin is expressed by neurons in the raphe nuclei of the brainstem which has many projections in the CNS (Descarries *et al.,* 1982; Sinton & Fallon, 1988; Hajós *et al.,* 1995). Studies have shown that injections of drugs which increase serotonin into the hypothalamus inhibit feeding and promote weight loss (Leibowitz & Alexander, 1998); in contrast, ablation of serotonergic neurons induces over eating known as hyperphagia (Nonogaki *et al.,* 1998). Not all cells within the raphe nucleus are serotonergic (Vertes & Crane, 1997), and other neurotransmitters may also mediate effects on food intake (Wirtshafter, 2001). Studies have shown that the catecholimines (dopamine, adrenaline and noradrenaline) have a varied effect on food intake. Injections of adrenaline into the raphe nucleus decreased energy intake and shortened meals in energy restricted rats, but increased feeding and meal number in free feeding animals, suggesting that adrenergic receptors in this particular nucleus are components of appetite-related neural circuits which control feeding initiation (dos Santos *et al.,* 2009).

### 1.1.9.3 Ventromedial Hypothalamic Nucleus

The VMH, one of the largest nuclei of the hypothalamus, has long been considered a ‘‘satiety centre’’. Stimulation of the VMH inhibits feeding, whereas lesions to this region cause overeating and weight gain (Kalra *et al.,* 1999). Recent studies have shown high abundance of leptin receptors (long form: *Ob-Rb*) in neurons of the VMH, and evidence indicates that this region may be an important target for circulating leptin (Williams *et al.,* 2001). Studies have shown that glutamatergic neurons, which secrete the excitatory amino acid glutamate, are abundant in the VMH, possibly implicating this amino acid in the communication of metabolic pathways (Collin *et al.,* 2003). An injection of glutamate directly into the hypothalamus causes an increase in feeding in satiated rats (Stanley *et al.,* 1993); this is due to the excitatory role of glutamate on orexigenic neurons (Elias *et al*., 2001). Dopaminergic activity in the VMH is involved in the control of meal number and inter-meal interval (IMI) (Meguid *et al.,* 1996; Fetissov *et al.,* 2002). The VMH has direct connections with the PVN, the LHA and the DMH.

### 1.1.9.4 Dorsomedial Hypothalamic Nucleus

The DMH, located immediately dorsal to the VMH, has direct connections with other hypothalamic nuclei such as the PVN, the LHA and the brainstem. The VMH and the LHA have no direct connections but connect indirectly through the DMH and the PVN. The PVN and the DMH may cooperate functionally as a unit, which is involved in initiating and maintaining food intake. The DMH contains numerous insulin receptors as well as leptin receptors (*Ob-Rb*). Some ARC NPY/AgrP neurons also terminate in the DMH (Williams *et al.,* 2001).

### 1.1.9.5 Lateral Hypothalamic Area

The LHA comprises a large, varying population of neurons including populations that express orexins and melanin-concentrating hormone (MCH), both peptides that stimulate food intake (Qu *et al.,* 1996; de Lecea *et al.,* 1998; Sakurai *et al.,* 1998). The LHA was viewed classically as the feeding centre. Stimulation of this nucleus increases food intake, while its destruction attenuates feeding and causes weight loss (Williams *et al.,* 2001). Dopaminergic activity in the LHA is involved in the control of meal size (Meguid *et al.,* 1996; Fetissov *et al.,* 2002). This nucleus also contains

large numbers of glucose-receptive neurons that respond to circulating glucose levels and are discussed further in later sections of this review.

### 1.1.10 Peripheral Appetite Signals

In addition to the hypothalamic peptides already discussed, there are numerous peripheral tissues which produce hormones and peptides that act on the brain, the ARC in particular, to control both short- and long-term feeding and energy expenditure.

### 1.1.11 Adiposity

There are several specific hormones that aid in the regulation of adiposity within the body. The main examples are described below.

#### 1.1.11.1 Insulin

The discovery of insulin led to the first indication that hormonal signals may be implicated in the regulation of body weight by CNS control mechanisms. Insulin is a peptide hormone secreted from the β-cells of the pancreas and accesses the brain through the blood acting on neurons in the ARC to reduce energy intake (Werther *et al.,* 1987). Furthermore, insulin receptors allow the transport of this hormone across the blood-brain barrier (BBB) of the brain and intracerebroventricular (i.c.v.) infusion of insulin results in suppression of food intake in a concentration dependent manner (Baskin *et al.,* 1999). Insulin is critically involved in the regulation of fat metabolism within the body as it causes liver, muscle, and fat tissues to absorb glucose from the blood whereby it is then stored as glycogen or triglycerides (Najjar, 2001). Insulin is present in the body in concentrations sufficient to remove glucose from the blood. When glucose concentrations drop, for example, just prior to a meal being taken, the body utilises stored sugar *via* the process of glycogenolysis which hydrolyses glycogen stored in the liver and muscle into glucose which can then be used as an energy source. If the control of circulating insulin concentration fails then the hormone becomes less effective at maintaining glucose homeostasis, which can result in the development of diabetes and this can have profound adverse effects on other physiological functions. Neurons are dependent on glucose as their energy source and a lack of this source can result in reduced function of the CNS (Ashcroft & Rorsman, 2012).

### 1.1.11.2 Leptin

Leptin is a hormone secreted from white adipose tissue (WAT) which circulates in direct proportion to the total amount of fat in the body (Zhang *et al*., 1994) and therefore, is known as an adipokine. It functions as part of a negative feedback mechanism to control energy intake, and hence, impacts on both adiposity and body weight regulation by crossing the BBB, acting on anorexigenic neurons in the ARC, and therefore inhibiting appetite (Berthoud, 2006). Leptin has an important role in the development of neurons within the hypothalamus (Bouret *et al.,* 2004; Pinto *et al.,* 2004). Studies have shown that the absence of receptors for leptin, for example in the *db/db* mouse, or the Zucker rat (*fa/fa*) results in uncontrolled feeding and obesity (Panchal & Brown, 2011). These leptin receptors are expressed in neurons of the ARC known to regulate energy balance (Håkansson *et al.,* 1998, 2000; Meister 2000). Elevated concentrations of leptin inhibit orexigenic neurons but stimulate anorexigenic neurons in the ARC nucleus to result in decreased food intake and increased energy expenditure. Leptin decreases food intake by opposing the effects of NPY and promoting the production of the appetite suppressant α-MSH (Pinto *et al.,* 2004). Currently, leptin and insulin are the only known adiposity regulators.

### 1.1.11.3 Adiponectin

Adiponectin is also an adipokine hormone secreted from white adipose tissue, however, unlike leptin it is inversely correlated with body fat accumulation (Scherer *et al.,* 1995; Fasshauer *et al.,* 2002; Viengchareun *et al.,* 2002). Circulating concentrations of adiponectin act on three receptors; AdipoR1, AdipoR2 and T-cadherin, to exert important functions in the control of glucose and lipid metabolism (Arita *et al.,* 1999; Yamauchi *et al.,* 2003; Kadowaki & Yamamuchi*,* 2005). Administration of recombinant adiponectin to rodents resulted in increased glucose uptake and fat oxidation in muscle, reduced hepatic glucose production, and improved whole-body insulin sensitivity (Kadowaki & Yamamuchi, 2005; Kadowaki *et al.,* 2006). In animal models of obesity and diabetes, treatment with adiponectin reduces blood glucose levels by hampering hepatic glucose production and increasing muscle glucose uptake. In addition, adiponectin increases free fatty acid oxidation in liver and muscle, which reduces the concentrations of free fatty acids in circulating plasma (Berg *et al.,* 2001; Yamauchi *et al.,* 2002; Karbowska &

Kochan, 2006; Yoon *et al.,* 2006). Studies have shown that i.c.v. administration of adiponectin into the hypothalamus provides an anorexigenic stimulus by activating the same signal transduction pathways as leptin and insulin (Coope *et al.,* 2008). In the hypothalamus, insulin acts in concert with leptin to provide the most robust adipostatic stimulus, informing the central nervous system about the peripheral energy stores and coordinating a complex neuron circuitry that maintains body energy homeostasis (Schwartz *et al.,* 2000). Adiponectin has a similar role inhibiting the orexigenic neurons of the hypothalamus.

#### 1.1.11.4 Ghrelin

Ghrelin is a hunger stimulating hormone produced by cells which line the stomach and is responsible for stimulating energy intake (Kojima *et al.,* 1999). Concentrations of circulating ghrelin increase before a meal, acting as signal of nutrient status in the gut, and decrease after a meal indicating energy sufficiency. The presence of ghrelin stimulates the production of growth hormone and ghrelin will bind to the growth hormone receptor present in the ARC nucleus of the hypothalamus and vagal-afferent endings in the gastrointestinal tract (Kojima *et al.,* 1999; Hewson & Dickson, 2000; Page *et al.,* 2007; Castañeda *et al.,* 2010). Ghrelin also activates cells in the ARC that include the orexigenic neuropeptide Y (NPY) neurons (Page *et al.,* 2007). There is also strong evidence that ghrelin has a peripheral appetite modulatory effect on satiety by affecting the mechanosensitivity of gastric vagal afferents, making them less sensitive to distension resulting in over eating (Kojima *et al.,* 1999; Hewson & Dickson, 2000; Castañeda *et al.,* 2010). In rodents, the expression of ghrelin is increased by energy restriction and weight loss, and i.c.v. infusion stimulates energy intake and body weight gain (Hewson & Dickson, 2000).

### 1.1.12 Satiety

Insulin and leptin are both involved in the longer-term regulation of energy intake, whereas short-term regulation is dependent on signals from the gastrointestinal tract which are generated in response to nutrient intake (McMinn *et al.,* 2000).

### 1.1.12.1 CCK

One of these hormones is cholecystokinin (CCK) which is released from the duodenum following the ingestion of fat and other dietary components, it aids in digestion and enhances satiety locally by acting on CCK-A receptors and centrally in the hypothalamus through CCK-B receptors (see Figure 1.7; Wilding, 2002; Dockray, 2004; Woods, 2004).

### 1.1.12.2 Peptide YY

Peptide YY (PYY) is another short-term regulating hormone secreted from cells that line the ileum and colon in response to the amount of energy consumed. PYY belongs to the same polypeptide family, as NPY and inhibits feeding by binding to NPY receptors in the ARC and therefore preventing action of the NPY neurotransmitter (see Figure 1.7; Batterham *et al.,* 2002).

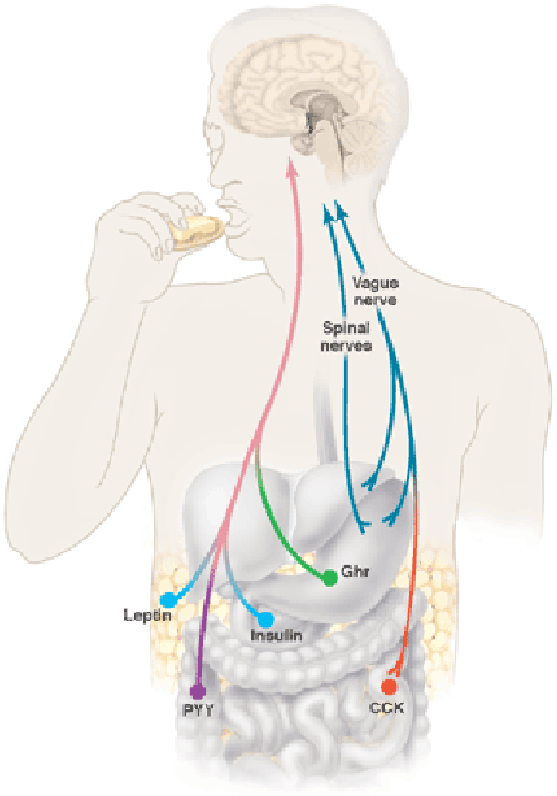



**Figure 1.7. Appetite controllers.** The body produces hormones that act through the brain to regulate short- and long-term appetite and metabolism. The diagram shows the sources of several of the hormones know to influence these control mechanisms. **Abbreviations**: CCK = cholecystokinin; Ghr = ghrelin, PYY = Peptide YY. Source: Marx, 2003. *Science* **299**(5608): 846-9.

### 1.1.13 Nutrient Sensing

Neurons sensitive to both glucose and fatty acids (FAs) are present in the hypothalamus and brainstem and play a key role in the CNS control of energy homeostasis.

#### 1.1.13.1 Glucose

Several mechanisms have evolved for detecting and regulating concentrations of plasma glucose and these are dictated by the presence of sensors located in a number of regions in the body, particularly the brain (Pénicaud *et al.,* 2000). Glucose is the primary energy source for the brain and is absorbed into the blood stream and crosses the BBB. The presence of neurons and glia sensitive to variations in glucose concentrations is well demonstrated in the brain, and in particular, in the hypothalamus (Levin, 2002; Pénicaud *et al.,* 2002). Glucose accesses the hypothalamus via the CSF in the 3V. Cell types which sense the presence of glucose contain specific transporters and channels which detect the presence of the refined sugar. There are two types of glucose-sensing cells: those that increase their activity when glucose increases (responsive) and those that decrease their activity under the same conditions (sensitive) (Levin, 2002). These cells are also involved in the control of food intake, energy expenditure and lipid metabolism (Pénicaud *et al.,* 2000). Short-term food intake is increased in response to low concentrations of circulating glucose (Mayer, 1955).

Glucose sensors are also present in different cell types throughout the body. The most commonly studied of these is the pancreatic β-cell where glucose sensors link plasma glucose variations to insulin secretion. These specific cell types express glucose transporter 2 (Glut2) and glucokinase. Glut2 facilitates a rapid glucose uptake irrespective of the extra-cellular concentration, whereas glucokinase controls glycolysis (Thorens, 2001). The cells within the brain which contain glucose sensors behave in a similar way (Jetton *et al.,* 1994; Leloup *et al.,* 1994) as confirmed by the presence of Glut2 (Jetton *et al.,* 1994; Roncero *et al.,* 2000; Thorens, 2001) and glucokinase in regions of the hypothalamus known to contain glucose sensitive neurons and glia (see Figure 1.8).

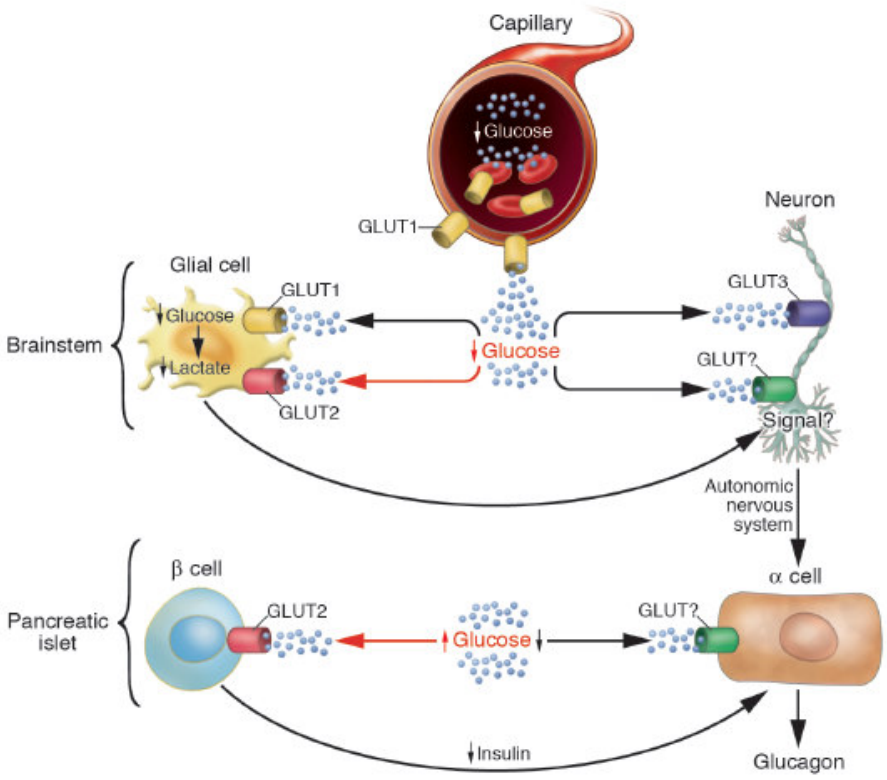



**Figure 1.8. Proposed glial-neuronal loop at work in central sensing of hypoglycaemia via GLUT2.** This scheme illustrates the pivotal role of GLUT2 in glial cells in first-hand detection of hypoglycaemia. How these specific glial cells then connect to neurons within the brainstem (NTS and dorsal motor nucleus of the vagus) to relay information is unknown but may involve the lactate shuttle as well as signalling via ATP-regulated $K^+$ channels (not illustrated). The drop in glycaemia may also be directly sensed by neurons and pancreatic α and β cells but not through GLUT2 (the transporter/detectors involved are so far unknown). Ultimately, autonomic nervous signals and the drop in intra islet insulin levels promote glucagon secretion. Source: Klip & Hawkins, 2005. *J Clin Invest* **115**(12): 3403–3405.

Glucose sensors are present in a number of brain regions. Specifically, responsive neurons are found in regions known for their involvement in the control of feeding, such as the VMH, ARC and PVN of the hypothalamus, the substantia nigra of the midbrain, the nucleus of the solitary tract, as well as the motor nucleus of the vagus in the brainstem (Dunn-Meynell *et al.,* 2002). Studies have shown that injecting an antisense against the glucose transporter, into the ARC of rats, results in a reduction in body weight (Levin, 2000). The most impressive effect of this treatment, however, is the complete inhibition of insulin secretion induced by an increase in blood glucose concentrations following an injection into the carotid artery (Leloup *et al.,* 1998). Other factors, such as fatty acids, hormones including insulin and leptin, and neurotransmitters, including NPY and orexin, also affect

glucose-responsive neurons (Yang *et al.,* 1999; Schuit *et al*., 2001; Williams *et al.,* 2001).

The hormones insulin and leptin and their respective hypothalamic receptors are responsible for the control of energy homeostasis by regulating food intake and energy expenditure. Studies have shown that insulin and leptin hyperpolarize rat hypothalamic glucose-responsive neurons by opening ATP sensitive potassium channels ($K_{ATP}$) (Spanswick *et al*., 2000a, 2000b). Therefore, glucose sensing neurons are part of a system which control energy homeostasis in an individual. However, studies have shown that these neurons are less abundant and function differently in models of metabolic disturbance such as obesity and type 2 diabetes (Rowe *et al.,* 1996; Spanswick *et al.,* 2000b; Levin, 2000; Song *et al.,* 2001; Levin, 2002).

Research has revealed changes in the brain of a rat model of obesity, induced by consumption of a highly palatable diet (Pickavance *et al.,* 2001). Interestingly, this diet incorporates only moderately elevated levels of macronutrients (fat and refined sugar), and therefore, is thought to exert its hyperphagic effects through the stimulation of hedonistic mechanisms, via "pleasure centres" within the brain, although this has yet to be proven. This model shows alterations in the size and character of specific populations of neurons in the hypothalamus and brainstem, which normally sense falling levels of glucose (glucose-sensitive) in the blood (Al-Qahtani *et al.,* 2008; Williams *et al.,* 2008). This has particularly important clinical implications; that obese people, with type 2 diabetes may be particularly vulnerable when treated with insulin or drugs designed to reduce glucose levels, as they may not be able to sense the induced hypoglycaemia, and therefore, trigger the counter-regulatory response. These, and many other, findings support a primary role for the brain in causing systemic dysfunction. The following section describes the role of fatty acid sensing in energy homeostasis.

### 1.1.13.2 Fatty Acids

Previously, it was thought that FAs were unable to cross the BBB; however, it has since been proven that lipids present in the brain are sourced from the plasma as well as by local synthesis (Rapoport *et al.,* 2001). This is likely due to the week integrity of the BBB in the region of the ARC/ME, although, some such as polyunsaturated

FAs (PUFAs) are able to cross the BBB regardless due to specific transporters (Edmond, 2001; Watkins *et al.,* 2001). FAs have been shown to activate neurons in the LHA (Oomura *et al.,* 1975) and modify firing rate of neurons in the ARC (Wang *et al.,* 2006). Studies have shown that i.c.v. infusion of the monosaturated FA (MUFA), oleic acid, decreases food intake, but replacing this FA with octanoic acid, a saturated fatty acid (SFA), had no effect, implicating the chain length and degree of saturation of the FA in this role (Obici *et al.,* 2002). The same effects on food intake can be seen when inhibiting hypothalamic FA oxidation, as inhibition results in an increase in acyl-CoA, an enzyme involved in the metabolism of FAs, which, in excess, will act as a satiety signal (Lam *et al.,* 2005). Studies showed that rats receiving a systemic or i.c.v. infusion of triglycerides (TGs) for two days increased plasma TG concentrations, which induced a decrease in sympathetic nervous activity. This decrease was responsible for the glucose-induced insulin secretion which characterises the pre-diabetic state (Magnan *et al.,* 1999) and hepatic insulin resistance (Clement *et al.,* 2002). The use of rodent models has also demonstrated that FA β-oxidation in the brain is required for their effects on insulin secretion and action (Cruciani-Guglielmacci *et al.,* 2004; Dowell *et al.,* 2005).

The hypothalamus can detect and respond to changes in circulating FA concentrations through the involvement of lipid-sensing neurons. These neurons use FA as a signalling molecule to regulate their membrane and action potential. Therefore, FA can modify ion channel activity and consequently induce changes in the secretion of neurotransmitters (Oishi *et al.,* 1990; Honen *et al.,* 2003). Studies have shown that there are oleic acid-sensitive neurons in the ARC which are excited by a closure of chloride channels, and inhibited by activation of $K_{ATP}$ (Wang *et al.,* 2006). However, oleic acid-sensitive neurons are insensitive to glucose concentrations. Therefore, it is suggested that among the neuronal population of the ARC some are sensitive to FAs and some to glucose. Yet, glucose concentrations seem to be involved in the function of FA-sensitive neurons (Pénicaud *et al.,* 2002). *In vitro* studies have shown that ARC neurons grown with steady-state glucose concentrations are mainly excited, whereas neurons maintained with low glucose concentrations are inhibited, following the administration of oleic acid. These findings suggest that the interaction between glucose and FAs regulates oleic acid-sensing in ARC neurons and that FA-sensitive neurons are activated or inhibited

dependent on the hypo-, normal, or hyperglycaemic state of an individual (Migrenne *et al.,* 2007). As FAs are known to decrease food intake in the short term, it can be expected that they act through the stimulation of anorexigenic (POMC/CART) and inhibition of orexigenic (NPY/AgrP) neuropeptide expression. Studies have shown that i.c.v. infusion of oleic acid inhibits glucose production and food intake by decreasing hypothalamic expression of NPY (Obici *et al.,* 2002).

### 1.1.14 Other CNS Centres and Systems

As mentioned previously, in the description of hedonic control of feeding, there are other CNS centres and systems involved in the control of feeding (see Figure 1.9). A brief overview of these additional regions is included below.

#### 1.1.14.1 Hippocampus

The hippocampus is not only central to memory, it is also involved in the regulation of feeding through its processing of mnemonic techniques; including remembering whether one ate, where food is located, identifying hunger and remembering how to relieve this state (Volkow *et al.,* 2011). The death of neurons within the hippocampus is linked with reduced cognition and memory as well as Alzheimers disease. Studies, using rodents, have shown that lesions of the hippocampus prevent the animal's ability to determine between the states of hunger and satiety (Davidson *et al.,* 2009), and in female animals resulted in hyperphagia (Forloni *et al.,* 1986). Human imaging studies have shown activation of the hippocampus in states of hunger (Haase *et al.,* 2009). The hippocampus expresses cannabinoid receptor type 1 (CB1) receptors, ghrelin and insulin all factors involved in the control of feeding regulation suggesting that the hippocampus may be involved in the regulation of energy intake via other homeostatic processes (McNay, 2007; Massa *et al.,* 2010).

#### 1.1.14.2 Reward Pathways

Reward pathways, such as those regulated by opioids and dopamine are heavily involved in the control of energy homeostasis. As mentioned previously, dopamine is a neurotransmitter which regulates reward-like behaviours through its projections from the ventral tegmental area into the nucleus accumbens, dorsal striatum, hippocampus and amygdala and LHA (Wise, 2006). Human studies have shown that the ingestion of palatable, energy dense foods results in an increase in dopamine

within the dorsal striatum in proportion to self-reported pleasure gained from eating the item (Small *et al.,* 2003). However, this can develop into a predictor of reward upon habituation to a food item and dopamine is then released upon anticipation (smell of food) rather than consumption (Epstein *et al.,* 2009; Schultz, 2010), resulting in the development of a conditioned response. The extensive glutamatergic afferents from dopamine neurons to regions involved in homeostatic (hypothalamus) and reward (nucleus accumbens) control regulate activity in response to varying food cues (Meguid *et al.,* 2000; Epstein *et al.,* 2007; Geisler & Wise, 2008). Mice with a dopamine deficiency die of starvation because of a lack of motivation to eat (Szczypka *et al.,* 2001), but restoration of this process prevents this. The hedonic properties of food also depend on endocannabinoid neurotransmission. Studies have shown that Tetrahydrocannabinol, the psychoactive constituent of the cannabis plant, acts via CB1 receptors in the hypothalamic nuclei to directly increase appetite. Hypothalamic neurons produce endocannabinoids that work to tightly regulate hunger (Berridge, 2009). The amount of endocannabinoids produced is inversely correlated with the concentration of leptin in the blood. Studies have shown that mice without leptin become obese but express high levels of hypothalamic endocannabinoids as a compensatory mechanism (Matias & Di Marzo, 2004).

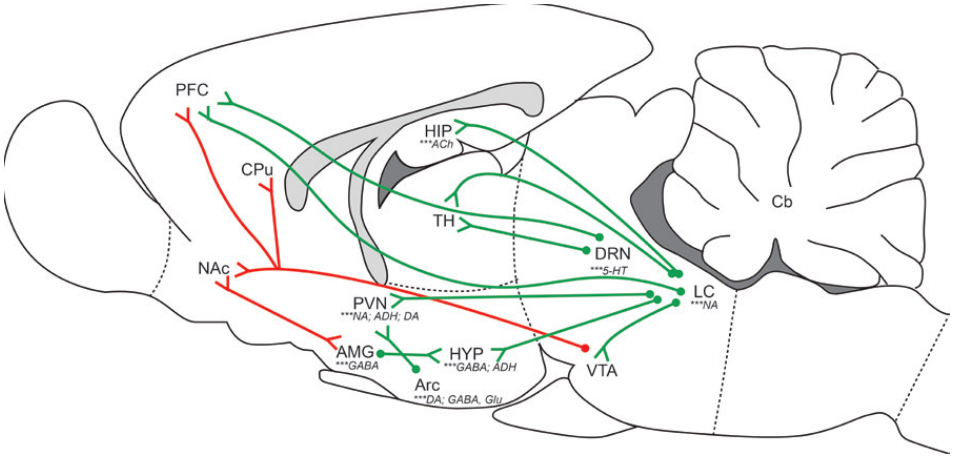



**Figure 1.9. Sagittal section through the rat brain depicting reward pathways (red and green lines).** Behavioural and anatomical observations suggest that nucleus accumbens to hypothalamus to hippocampal projections are involved in the reward-driven food intake by modulating hypothalamic feeding circuits, thought to be crucial for homeostatic control of energy balance. Indirect projections though the ventral pallidum and projections from the amygdala and relevant cortical areas may also be important. **Abbreviations:** 5-HT, serotonin; ACh, acetylcholine; ADH, antidiuretic hormone; AMG, amygdala; Arc, arcuate hypothalamic nucleus; Cb, cerebellum; CPu, caudate putamen (striatum); DA, dopamine; GABA, gamma-aminobutyric acid; Glu, glutamate; HIP, hippocampus; HYP, hypothalamus; LC, locus coeruleus; NAc, nucleus accumbens; NA, noradrenaline; PFC, pre-frontal cortex; PVN, paraventricular hypothalamic nucleus; TH, thalamus; VTA, ventral tegmental area. Source: Ash & Djouma, 2011. *J Addict Res Ther* **4**:003.

Many of the feeding related control mechanisms described above display elements of circadian rhythm. This clock-like control mechanism is controlled by several of the hypothalamic nuclei mentioned previously, as well as the suprachiasmatic nucleus (SCN).

### 1.1.15 Circadian Rhythms

Many of the biological functions which are essential for an individuals' needs demonstrate a rhythmic pattern which tracks the 24-hour clock. These daily variations are called circadian rhythms and allow for the preparation of environmental changes such as the diurnal (light) and nocturnal (dark) phases and associated food availability. Hence, the timing of many metabolic, behavioural and physiological processes is controlled by this circadian clock in association with any environmental changes. In mammals, this circadian system is comprised of several endogenous clocks. The main clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Welsh *et al.,* 1995). Neurons present in this nucleus receive direct input from the retina in order to synchronise with changes in light, i.e. from diurnal to nocturnal phase (Meijer & Schwartz, 2003). When the presence of light activates the receptors of the retina, nerve impulses are conducted *via* the retino-hypothalamic tract directly to the SCN (Hattar *et al.,* 2002) which allows for the rhythmic control of metabolism via neuronal connections and circulating factors, within a 24-hour time period (Figure 1.10). Circadian clocks function because of specific genes known as clock genes. Clock genes are expressed in almost all tissues and their products interact at the transcriptional level to generate circadian oscillations (Ko & Takahashi, 2006).

### 1.1.16 Circadian Control of Metabolism

There are several hypothalamic nuclei which receive direct neuronal input from the SCN. These include the PVN (Dibner *et al.,* 2010), which projects to peripheral tissues, such as the liver (Shibata, 2004), pancreas (Buijs *et al.,* 2001) and adipose tissue (Kreier *et al.,* 2002) via autonomic pathways. Furthermore, the VMH, LHA and ARC contain glucose-sensing neurons which receive nutritional information from glucose and hormones, including leptin and insulin in the plasma, and neuronal messages from the brainstem, including the nucleus of the solitary tract (NTS). These circulating factors are all known to influence energy homeostasis, and

concentrations follow a 24-hour pattern (Kalsbeek *et al.,* 2001; Yamamoto *et al.,* 1987). Additionally nonesterified fatty acids in the liver also follow this pattern, thereby demonstrating that the SCN regulates the different clock genes in central and peripheral tissues.

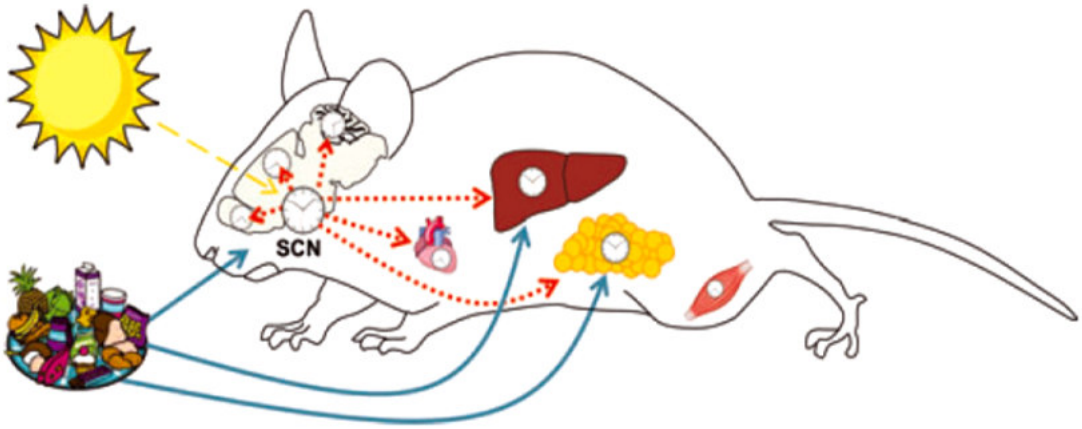



**Figure 1.10. Organization of the circadian timing system.** The master clock, located in the suprachiasmatic nucleus (SCN) of the hypothalamus, adjusts the timing of many secondary clocks/oscillators in the brain and peripheral organs, in part via nervous pathways (dotted red lines). Light perceived by the retina is the most potent synchronizer of the SCN clock (dashed yellow line); while meal time can synchronize peripheral clocks (blue arrows). Source: Delezie & Challet, 2011. *Ann N Y Acad Sci* **1243**: 30-46.

In rats, ablation of the SCN removes the circadian pattern of food intake (Nagai *et al.* 1978). While the master clock in the SCN is mainly synchronized by light, clock gene oscillations in peripheral tissues can be shifted by feeding time (Stokkan *et al.,* 2001; Feillet *et al.,* 2006). However, in conditions of extreme energy restriction, the synchronization of the SCN to light can be modified (Challet, 2010); this is potentially due to feedback from peripheral hormone signals confirmed by the presence of insulin, leptin and ghrelin receptors in the SCN (Håkansson *et al.,* 1998; Zigman *et al.,* 2006). This reinforces the role of the circadian system in the daily variation seen in parameters of metabolism (Delezie & Challet, 2011).

Studies have shown that in the *ob/ob* mouse alterations in peripheral clocks occur before the onset of obesity and hyperinsulinaemia, suggesting that the clock can play a role in obesity in addition to the leptin deficiency. Within the SCN of these mice, the molecular clockwork is unchanged (Ando *et al.,* 2011). Short-term high-fat feeding reduces circadian variations of leptin concentrations in rats (Cha *et al.,* 2000) and humans (Havel *et al.,* 1999) which could contribute to the

development of obesity. In mice, high-fat feeding attenuates the daily pattern of food intake, with a higher consumption during the day and a decrease during the active period, long before any significant weight gain is observed (Delezie & Challet, 2011). Furthermore, changes in concentration and temporal pattern of glucose, insulin, leptin, and nonesterified fatty acid expression were also observed (Kohsaka *et al.* 2007). Diets high in fat seem to have a direct effect on the SCN as shown by studies where mice fed high-fat diets had disrupted synchronization to light (Mendoza *et al.,* 2008).

In clock gene, knockout mice, the development of hyperphagia is associated with an increased food intake during the diurnal/light phase, and attenuated energy expenditure at night, thus contributing to body weight gain in excess. Furthermore, these mice displayed hypertriglyceridemia, hyperglycemia, increased insulin sensitivity and altered gluconeogenesis (Rudic *et al.,* 2004). In addition, there were also changes in the expression of hypothalamic neuropeptides, like CART and orexin, which play a role in central circuits controlling feeding and pleasure (Turek *et al.,* 2005). Therefore, in summary, impairment of clock gene oscillations and metabolic pathways may explain the altered coordination of metabolic functions and clock-controlled output signalling, contributing to obesity and associated disorders.

### 1.1.17 Definition of Obesity

Obesity is a chronic metabolic disease resulting from an imbalance between energy intake and energy expenditure. The defining feature of obesity is an elevated body weight, due to an abnormal level of adiposity (Schwartz & Brunzell, 1997; Seidell & Flegal, 1997). White adipose tissue is the major site for storage of excess energy in the body. It is composed of adipocytes, extracellular matrix, vascular and neural tissues, preadipocytes, fibroblasts, stem cells, and immune cells such as macrophages and T lymphocytes. Adipose tissue secretes numerous peptides collectively known as adipokines such as leptin and adiponectin (described previously), and inflammatory cytokines such as Interleukin 6 (IL-6), Interleukin 1 (IL-1) and Tumor necrosis factor-alpha (TNF-α). Thus, adipose tissue is a dynamic endocrine organ with major roles in energy balance, glucose homeostasis and immune function. Excessive triglyceride (TG) accumulation within adipocytes, as a result of a positive energy balance (Figure 1.3), leads to adipocyte hypertrophy and a dysregulation of

adipokine secretory patterns. This has been primarily attributed to an imbalance in inflammatory adipokines. Thus, obesity is associated with a chronic low-grade inflammation in the adipose tissue (for a review see Kalupahana *et al.,* 2011). Major cell types that play key roles in the inflammatory response during the onset of obesity can be seen in Figure 1.11. Obesity is considered a disorder of low-grade chronic inflammation, both peripherally, in the adipose tissue, and centrally, in the hypothalamus, as an increase in circulating TGs activates hypothalamic inflammatory signalling pathways, resulting in increased food intake and nutrient storage (Lumeng & Saltiel, 2011).

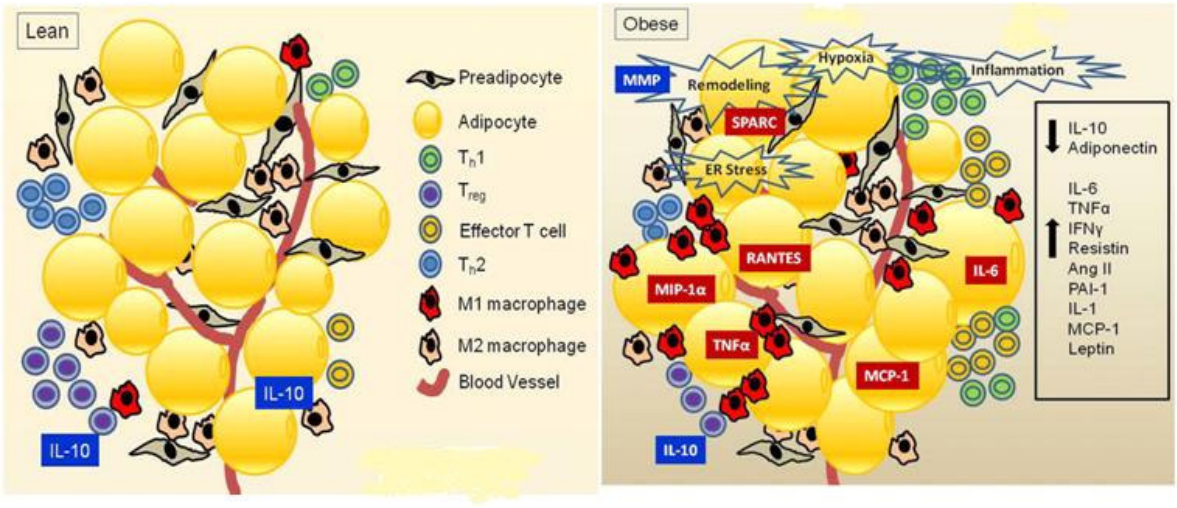



**Figure 1.11. Obesity-associated immune cell infiltration of adipose tissue.** Lean individuals exhibit higher ratios of M2:M1 macrophage, $T_h1$:$T_h2$ T cell, and regulatory effector T cell. Excessive TG accumulation leads to adipose tissue remodelling, relative hypoxia, and endoplasmic reticulum (ER) stress, which trigger production of chemokines and changes in the above cell ratios, culminating in increased production of proinflammatory adipokines and reduced production of anti-inflammatory adipokines. **Abbreviations**: Ang II, Angiotensin II; IFN-γ, Interferon-gamma; IL-1, Interleukin 1; IL-6, Interleukin 6; IL-10, Interleukin 10; MCP-1, monocyte chemotactic protein-1; MIP-1α, Macrophage Inflammatory Proteins; MMP, Matrix metalloproteinases; PAI-1, Plasminogen activator inhibitor-1; RANTES, regulated and normal T cell expressed and secreted; TNF-α, Tumor necrosis factor-alpha. Source: Kalupahana *et al.* 2011. *Adv Nutr* **2**:304-316.

Obesity is caused by the interaction of multiple genetic and environmental factors (Vandenbroeck *et al*., 2007) and at present there is a lack of evidence for effective preventative measures (Brown *et al*., 2007). Research clearly indicates that human biology gives many individuals an underlying tendency to accumulate energy and conserve it because of factors such as genetic risk (Farooqi & O'Rahilly, 2007), the influence of early life experiences (Simopoulos, 1987; Schwartz & Brunzell,

1997; Seidell & Flegal, 1997; Barker, 2007; Singhal & Lanigan, 2007) and the sensitivity of the appetite control system (Bloom, 2007; Rolls, 2007; Wardle, 2007). However, these factors alone are unable to fully account for the recent rapid increase in the prevalence of obesity in the population, and this is likely to be the result of many biological, social and environmental factors including changes in food production and lifestyle patterns such as lack of exercise. These changes have resulted in an 'obesogenic environment' which has detrimental effects for the human population.

The clinical assessment of body fat is approximated using a formula that combines an individual's weight and height. This is known as the body-mass index (BMI), which is the weight of a person in kilograms divided by the square of their height in meters. Because of differences in body composition among the human population, the measure of BMI may not always accurately correspond to the same degree of fatness, but it is currently the most commonly used method for calculating it. An individual with a BMI between 20 and 24.9 is classified as 'normal', between 25.0 and 29.9 is classified as overweight, and a person with a BMI above 30.0 is classified as obese. In extreme cases where BMI is over 40, a person is classified as morbidly obese (Kopelman, 2000).

### 1.1.18 Risk Factors for Obesity

Obesity develops because of an imbalance in energy intake and expenditure, as a result of behaviours (feeding and time spent active) and physiology (resting metabolism and expenditure when active), both of which are affected by genetic and environmental factors. The dramatic increase in the numbers of obese people in current society reflects changes in environmental factors such as increased food intake and reduced physical activity. However, in all populations some individuals will remain lean. These differences are likely as a consequence of genetic factors as is supported by the high heritability of body composition (Speakman, 2004).

#### 1.1.18.1 Environmental Factors

The prevalence of overweight and obese individuals has increased dramatically in the last few decades. It is easy to identify some of the factors influencing this problem. One of these factors is an increase in the number of hours spent watching

the television (Armstrong *et al.,* 1998; Crawford *et al.,* 1999; Hernandez *et al*., 1999; Dennison *et al.,* 2002; Janz *et al.,* 2002; Lowry *et al.,* 2002) and the negative impact this has on the hours spent conducting physical activity (Janz *et al.,* 2002; Eisenmann *et al.,* 2002). Many other changes in physical activity patterns have also changed, for example most people will now use the car to drive to a large supermarket rather than walk to their local shops (Eisenmann *et al.,* 2002). At the same time as changes in level of physical activity have occurred, dietary habits have also altered. These alterations include the increased consumption of meals away from the home (Binkley *et al.,* 2000) and greater consumption in total energy intake through snacks, sugary drinks, and fatty and fast food items (Nielsen *et al.,* 2000; Nielsen & Popkin, 2003). Insights in neurobiology show how powerfully the wide variety and appeal of modern foods, with their increased palatability and capacity to heighten sensory stimulation, drive us to reward ourselves with the consumption of more food (Chipperfield *et al*., 2007; Jackson & Harris, 2007; Maio *et al*., 2007; Rolls, 2007; Vandenbroeck *et al*., 2007; Wardle, 2007). Therefore, combinations of reduced physical activity and increased energy consumption are likely responsible for the rise in obesity.

#### 1.1.18.2 Genetic Factors

Despite the increase in the number of overweight and obese individuals, not everyone fits this category. This effect can be traced to individual differences in behaviour and physiology. These behavioural differences have a large genetic component to them. Therefore, despite individuals being exposed to similar environmental factors some people will develop obesity because their genetic makeup is such that they are more likely to adopt behaviours, or physiologies (such as low BMR), that predispose them to a positive energy balance (Speakman, 2004).

#### 1.1.18.3 Monogenic Forms of Obesity

Studies have shown that obesity can, though rarely, develop as a result of one single gene mutation (Loos & Bouchard, 2003). For example, a small number of children have been found to have a leptin mutation. The phenotype of these individuals is very similar to the *ob/ob* mice which are obese, hyperphagic and hyperinsulinaemic (Montague *et al.,* 1997). Some of these children were treated with recombinant leptin for a year and showed a reduction in weight and food intake as well as a normalising

of circulating hormones (Farooqi *et al.,* 1999). Another example of a single gene mutation resulting in obesity is obvious in individuals with POMC deficiency syndrome characterised by early-onset obesity, hyperphagia, hypocortisolaemia and red hair with pale skin due to a reduction in melanocortin receptors (Farooqi *et al.,* 2006). The rodent homologue is the agouti ($A^y$) mouse which is obese with yellow fur, the mice exhibit mild hyperphagia and hyperglycemia (Manne *et al.,* 1995). There are other rodent models of monogenic forms of obesity including the *db/db* mouse, Zucker and ZDF rats.

### 1.1.18.4 Polygenic Forms of Obesity

Individual cases of obesity caused by a single-gene mutation are rare; however, interactions between the polygenic variants in a range of genes are more common. Any of a group of alleles at distinct gene loci that collectively control a phenotype is termed a 'polygenic' variant. A polygenic effect can only be identified by showing that such an allele occurs in greater numbers in an obese individual than their lean counterpart. Potentially, many such polygenic variants play a role in body weight regulation. If an individual harbours many polygenic variants that increase body weight, obesity can ensue. Any single variant will have a higher frequency in obese than in normal weight and lean individuals. A polygenic basis of obesity implies that the specific set of polygenic variants relevant for obesity in one individual is unlikely to be the same in another obese subject (Hinney & Hebebrand, 2008). When researching the nature of polygenic obesity, many studies have selected candidate genes on the basis of their involvement in pathways of energy balance or adipose tissue biology. Both transgenic knockout mice and human studies have identified the leptin gene as a candidate, as increases in expression of this gene show positive associations with body weight, weight loss and BMI. Association studies between obese phenotypes and other key proteins (POMC, AgrP and NPY) are limited and require replication (Hinney *et al.,* 2010). Genome-wide scans have identified evidence that the chromosome 2p22 is linked with both leptin and adiponectin influence on obesity (O'Rahilly *et al.,* 2003). The modelling of polygenic obesity in rodents is discussed in Chapter 2 Section 2.1.2.2. Subtle differences between obese phenotypes and additional effects of the environment make characterising the genetic basis of obesity quite difficult (Figure 1.12).

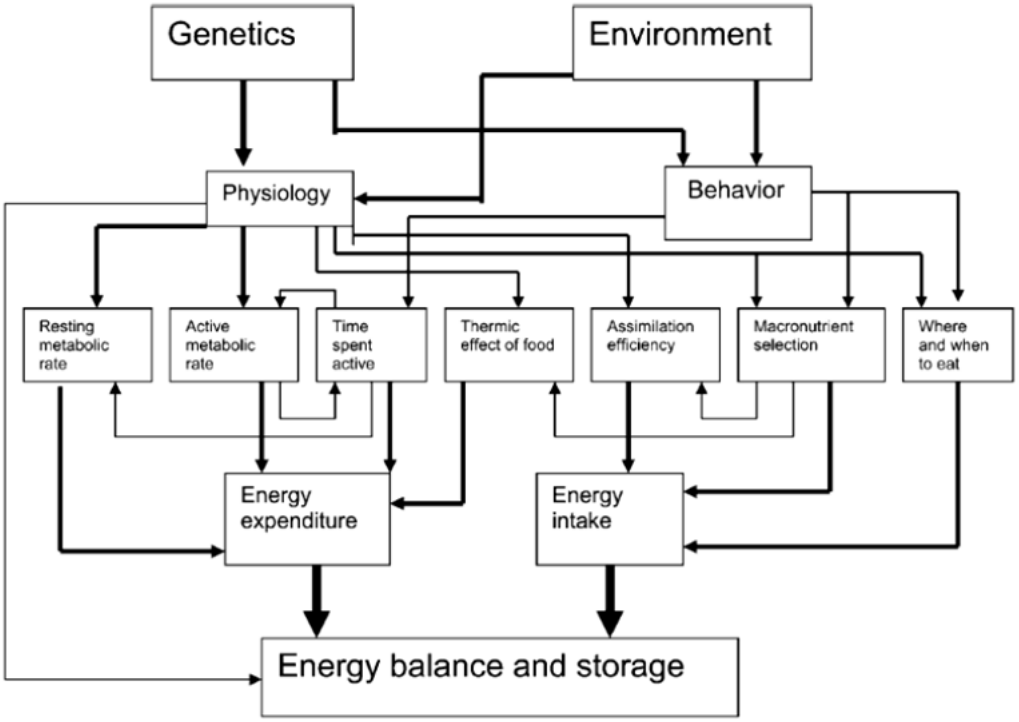



**Figure 1.12. The major causal linkages among genetics, environmental effects, physiology, behaviour, and energy balance.** Multiple causal routes exist by which both environmental and genetic effects may exert their influence. The diagram clarifies that behaviour is not an alternative mechanism to genetics; they are different levels of the same phenomenon. Source: Speakman, 2004. *J Nutr* **134**(8): 2090-2105.

### 1.1.19 Significance of Obesity

#### 1.1.19.1 Obesity as a Medical Problem

Obesity is a major worldwide health issue which dramatically increases the risk of developing several disorders, including impaired glucose tolerance, insulin resistance, type 2 diabetes, cardiovascular diseases, dyslipidaemia, arthritis, gallstone formation (Mokdad *et al.,* 2001; Lobstein *et al.,* 2007), attendant disorders including cancers, such as breast, colon, uterus, pancreas, and kidney cancer (Calle *et al.,* 2003; Kaidar-Person *et al.,* 2011) and cognitive impairment and neurodegenerative disease e.g. Alzheimer's disease (Doherty, 2011).

#### 1.1.19.2 Type 2 Diabetes

In instances of diabetes during obesity, fasting insulin concentrations are elevated, resulting in an increased insulin response to a glucose load. The enlarged presence

and distribution of adipose depots influences glucose metabolism, increasing apoptosis and thus, the release of free fatty acids from abdominal adipocytes, resulting in a greater uptake of insulin by the liver and finally, in increased gluconeogenesis and dyslipidaemia. These changes then lead to the development of hyperinsulinaemia and decreased skeletal insulin sensitivity. The inability of β-cells to adapt, long term, to this situation, and produce more insulin, leads to the development of type 2 diabetes. Studies have shown that even a relatively small weight gain of 5 kg increases the risk of diabetes by 50%, while losing the same amount of weight reduces the risk by the same (Kokkoris & Pi-Sunyer, 2003).

#### 1.1.19.3 Neurodegenerative Conditions

Furthermore, obesity also increases the risk of neurodegenerative conditions such as dementia, Alzheimer's, and Parkinson's disease, which threaten quality of life and shorten life span. These diseases are characterised by the loss of structure, function, and death of neurons and are often associated with ageing. Leptin resistance or insensitivity often develops in individuals suffering from obesity, and in addition, the leptin signalling system is less active in the elderly (Doherty, 2011). These findings, and those that demonstrate that leptin has beneficial effects on both the survival and neurophysiology of the neurons that are lost in Alzheimer's disease, reveal that this particular hormone may be a link between neurodegeneration and obesity or ageing (Doherty, 2011). Furthermore, obesity is associated with an increased consumption of diets high in fats and refined sugars; these dietary components are also associated with cognitive impairment within the hippocampus (Stranahan *et al*., 2008); a region known for its control of memories and learning. Studies have shown that increased consumption of diets high in these two items results in hyperphagia and obesity by interfering with hippocampal dependent memory inhibition that is critical in the ability of animals to refrain from responding to environmental cues associated with food, and ultimately from consuming energy intake in excess of that driven solely by essential need (Kanoski & Davidson, 2011).

### 1.1.20 Socioeconomic Significance of Obesity

#### 1.1.20.1 Burden on the NHS

Dealing with the long-term consequences of obesity costs an estimated £5.1 billion each year (Scarborough *et al*., 2011), placing a huge strain on the NHS. Furthermore, the NHS has had to invest substantial sums of money in specialised facilities and technologies for handling morbidly obese patients, including lifting equipment, reinforced surgical tables and bariatric ambulances (Scarborough *et al*., 2011). Levels of obesity in the UK are increasing, with over a quarter of the adult population now classed as obese (HSCIC, 2012). It is estimated that these figures could reach 60% for men, 50% for women and 25% for children by 2050 (McPherson *et al.,* 2007). As a consequence, the proportion of associated disease, such as type 2 diabetes and metabolic syndrome will also increase substantially (Kopelman, 2007; McPherson *et al.,* 2007). Expert opinion has suggested that, in the absence of additional interventions, the prevalence of obesity will continue to rise with no expectation of any reversal (Chipperfield *et al.,* 2007). Therefore, the developments of both pharmaceutical and natural interventions which protect against this dysfunction are important to human health (Dourish *et al.,* 2008; Buckley & Howe, 2009) as well as the economy.

#### 1.1.20.2 Economic Impact

In addition to the well-documented health impacts, obesity also leads to a loss of individuals in the work force (Johnson *et al.,* 2008). Obese workers tend to have higher rates of absenteeism and take more sick leave therefore increasing costs for the employer and reducing productivity (Neovius *et al.,* 2009). Furthermore, specialist equipment, such as wider chairs, must be purchased to accommodate obese workers (Bakewell, 2007). Studies have shown that employees with a BMI of 40+ (morbidly obese) regularly have many more accidents such as falls at work, resulting in claims, than employees with a BMI of 18-25 (Ostbye *et al.,* 2007). Due to the ever increasing rates of obesity, airlines currently face higher fuel costs and pressures to increase seating width (Dannenberg *et al.,* 2004).

#### 1.1.20.3 Growing Prevalence in the Developing World

Until the last few decades, incidences of obesity were rare (Haslam, 2007). In 1997, the world health organisation (WHO) officially recognised obesity as a global epidemic. In 2005 WHO estimated that over 400 million adults, approximately 10% of the total population at that time, were obese, with greater prevalence among females (Caballero, 2007). Obesity rates in Australia, Canada and the USA are currently increasing at greater rates than the overall worldwide rate (Howard *et al.,* 2008). Previously, obesity was considered as a problem for developed countries only, but now incidences are increasing worldwide (Tsigos *et al.,* 2008). Currently, sub-Saharan Africa is the only remaining region where obesity is still uncommon (Haslam & James, 2005).

### 1.1.21 Treatment of Obesity

Currently, there are limited options for treating obesity, and regimes including dieting and increased physical exercise (see Figure 1.2), as a combination with behavioural modifications are commonly recommended. A combined intervention consisting of a reduced-energy diet, increased physical activity and behavioural modification is the most effective therapy for weight loss and maintaining that loss. Weight loss occurs by generating a negative energy balance, which is achieved by consuming less energy (energy in), than that expended (energy out) (Figure 1.2; Blackburn & Walker, 2005; Tate, 2007). As a last resort there are also pharmaceutical and surgical interventions (Rucker *et al.,* 2007). Surgical interventions such as the insertion of a gastric band or gastric bypass may assist with weight loss, and/or the surgical reduction of stomach volume and bowel length may lead to effects of increased satiation and reduced ability to absorb nutrients from food consumed (Mathus-Vliegen & Tytgat, 2005; Imaz *et al.,* 2008). However, side effects include deficiencies in vitamins, iron, calcium and electrolytes (Korner *et al.,* 2006).

#### 1.1.21.1 Pharmaceutical Intervention

Anti-obesity pharmaceuticals may also be taken to decrease appetite and/or inhibit the absorption of fat into the body but these are intended to be taken in conjunction with a suitable diet and physical activity regime. Over the years there have been

around 120 different types of anti-obesity medications; however, currently there is only one licensed medicine available, known as Orlistat. It works by preventing the digestion of fat, so it is not absorbed into the body, and is passed out in faeces. This medicine helps to prevent weight gain but must be combined with diet and exercise regimes to aid in weight loss and even then, weight loss may be slow (Rucker *et al.,* 2007). Doctors will only prescribe this drug when a significant attempt at weight loss through diet and exercise has been demonstrated and the patients BMI is 30 plus or, if the patients BMI is 27 plus and they have an associated condition of obesity such as diabetes or heart problems. There are a number of side effects associated with this drug, they include oily discharge and oily stools, urgent and frequent passing of stools, flatulence, stomach cramps, headaches and respiratory tract infections (Rucker *et al.,* 2007). In July 2012, two other medications were also approved for use by prescription only; these include lorcaserin and a combination pill of phentermine and topiramate (Bays, 2011a, b). Lorcaserin is a selective 5-$HT_{2C}$ receptor agonist and activation of these receptors in the hypothalamus activates POMC production and therefore promotes weight loss through satiety (Thomsen *et al.,* 2008). In comparison, phentermine is an appetite suppressant and achieves this through stimulation of the amphetamine and phenethylamine classes. Topiramate is an anticonvulsant that has weight loss side effects due to its ability to reduce appetite (Hellmich, 2012).

There are numerous side effects associated with taking these anti-obesity drugs and many have been removed from the market due to serious issues such as depression, suicide, cardiovascular problems and birth defects (Rucker *et al.,* 2007).

#### 1.1.21.2 Relative Success

Currently, there is no simple cure for obesity. The management of numerous external factors have to be considered when evaluating obesity as a whole. Necessary adjustments are required to dietary habits, physical activity and behavioural traits. Furthermore, drug treatment is currently available, but retrospectively it has mostly been a disappointing proponent in the initiative against obesity due to detrimental side effects. Anti-obesity drugs are able to assist in short-term weight loss but are unsatisfactory when considering maintaining a lower weight. Surgical interventions are available but even they have negative side effects.

#### 1.1.21.3 Potential for Nutraceuticals

As the prevalence of obesity is continuing to rise (Chipperfield *et al.,* 2007) and current pharmaceutical treatments are lacking, the development of natural interventions which protect against this dysfunction are important to human health (Dourish *et al.,* 2008; Buckley & Howe, 2009).

#### 1.1.21.4 Nutraceuticals

Nutraceuticals are food items that provide health and medical benefits (Wildman, 2001) and these products are expected to prevent chronic illnesses, improve health and increase life expectancy (Kalra, 2003). In the case of obesity, the preference for research of nutraceuticals over pharmaceuticals is clear due to a lack of available drugs to treat the condition.

#### 1.1.21.5 Examples of Nutraceuticals

There are several examples of food items that have medicinal properties; these include the antioxidants which prevent oxidation of molecules into free radicals which can cause cell damage, tumour formation and cancer (Rajamani *et al.,* 2011). Examples of beneficial antioxidants include resveratrol from red grape products, flavonoids inside citrus fruits, tea, red wine, and dark chocolate, and anthocyanins found in berries (Aggarwal, 2010). Dietary fibre has also been shown to reduce hypercholesterolemia (Weingartner *et al.,* 2008), and broccoli, which contains sulforaphane, is thought to have anti-cancer properties as the sulforaphane enhances the expression of tumour suppressor proteins (Hayes *et al.,* 2008). Furthermore, the isoflavonoids found in soybean are thought to improve arterial and cardiac health (Aggarwal, 2010). One of the most versatile and well recognised of the nutraceutical families are the omega-3 polyunsaturated fatty acids (PUFAs) found in fish oil.

#### 1.1.21.6 Omega-3s as Nutraceuticals

The omega-3 PUFAs are precursors for the eicosanoids (DeCaterina & Basta, 2001), which are signalling molecules that act as vasodilators and bronchodilators in conditions such as asthma and heart disease (Funk, 2001; Judé *et al*., 2006). These FAs may also prevent the activity of the enzyme tyrosine kinase (Carpentier *et al.,* 2006), which is involved in the cell-growth signalling mechanism and this reduces

cardiac hypertrophy and the rapid cell proliferation involved in cancer formation (Wildman, 2001). PUFAs also appear to inhibit the synthesis of fatty acid synthase, which is the principal enzyme involved in fatty acid synthesis (Buckley & Howe, 2009). This leads to decreased quantities of body fat reducing the likelihood of obesity and therefore, indirectly reducing the development of associated conditions such as hyperinsulinaemia, hypertension and hyperlipidemia (Wildman, 2001).

Alpha-linoleic acid (omega-6 PUFA) from flax and omega-3 fatty acids present in fish oil are already well established as reducing the risk of cardiovascular disease (Shibamoto *et al*., 2008; Weingartner *et al.,* 2008). However, they are also known to prevent cognitive decline, dementia (Cunnane *et al.,* 2009), macular degeneration (Poudyal *et al.,* 2011), breast, prostate, and colorectal cancer (Rose & Connolly, 1999), inflammatory disorders, arthritis and inflammatory bowel conditions (Sijben, 2009). They are also involved in promoting brain cognition and development as well as visual acuity (Poudyal *et al.,* 2011). Dietary omega-3 PUFAs are also important for neonatal health and development (Horrocks & Yeo, 1999; Innis, 2000; Voigt *et al*., 2000) in addition to the generation of a healthy immune system (Sijben, 2009). The structure and function of the omega-3 PUFAs is discussed in later sections of this review.

#### 1.1.21.7 Health Benefits Associated with omega-3 Fatty Acids

Studies have shown that dietary modifications, including an increase in the relative abundance of omega-3 PUFAs in the diet, can protect against metabolic disturbances (Yashodhara *et al*., 2009). The cardioprotective effects of omega-3 fatty acids, especially Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA), have been well defined by cell culture, animal and human studies (Anderson, 2009; Duda *et al*., 2009; Lee *et al.,* 2009; Saremi *et al.,* 2009; Yashodhara *et al*., 2009). The main source of EPA and DHA in the human diet is from fish (Lee *et al*., 2009), which acquire these PUFAs by consuming algae rich in linoleic acid which is then converted into the longer and more unsaturated forms (Wildman, 2001). There is considerable evidence, through studies involving rodents, that supplementing a diet high in fat with omega-3 PUFAs can attenuate weight gain and reduce body fat, particularly the epididymal depot, as well as reducing circulating plasma TGs (Cunnane *et al*., 1986; Belzung *et al.,* 1993; Hainault *et al*., 1993; Baillie *et al.,* 1999; Ruzickova *et al*., 2004). The impact of consuming omega-3 PUFAs on body

composition has yet to be fully determined in the human population but research has shown that increasing consumption of omega-3 PUFAs by approximately 3 g per day can also reduce body weight and body fat in the overweight and obese (Kabir *et al*., 2007).

### 1.1.21.8 Mechanisms behind Health Benefits

Increasing the dietary intake of omega-3 PUFAs may assist with body weight and body fat reduction by several mechanisms. These include altered gene expression (activate AMP-activated protein kinase), resulting in increased fat oxidation in adipose, liver, cardiac, intestinal and skeletal muscle tissue and reduced fat deposition in adipose tissue (Buckley & Howe, 2010; Kalupahana *et al.,* 2011). Omega-3 PUFAs also increase satiety therefore preventing hunger, although most studies, including those in rodents, have not observed reduced food intake (Cunnane *et al*., 1986; Hainault *et al*., 1993; Hill *et al*., 1993). Results from some studies have indicated that omega-3 PUFAs may promote an increase in lean body mass, therefore increasing metabolic rate and decreasing body fat (Buckley & Howe, 2009). Discrepancies in the results of many studies which administer PUFAs to rodents make it difficult to draw valid conclusions on their exact beneficial roles. More systematic studies need conducting in order to overcome this problem. This concept is discussed in more detail in Chapter 2.

PUFAs are natural ligands at the key transcription factors, the retinoic acid receptors (RARs), retinoid X receptors (RXRs) and the peroxisome proliferator-activated receptors (PPARs), and in this way directly regulate gene transcription related to a range of functions, including energy metabolism and cognition. By this mechanism, they act at the level of the adipocyte to attenuate the accumulation of adipose tissue by inhibiting expression of lipogenic genes and stimulating transcription of those involved in lipid oxidation (see Figure 1.13; Bordoni *et al.,* 2006). Omega-3 PUFAs modulate adipokine secretion from adipose tissue. They increase plasma adiponectin levels in obese humans and rodents (Flachs *et al.,* 2006), which could be a potential mechanism by which they improve insulin sensitivity. They also induce leptin secretion and reduce the expression of several inflammatory cytokines from the adipose tissue, including TNFα and IL-6 (Kadowaki *et al.,* 2006). Production of inflammatory cytokines is dependent on

activation of the NF-$_k$B and JNK pathways. Omega-3 PUFAs bind to G protein-coupled receptor (GPR) 40 or 120, and inhibit NF-$_k$B and JNK, attenuating this response (Kalupahana *et al.,* 2011).

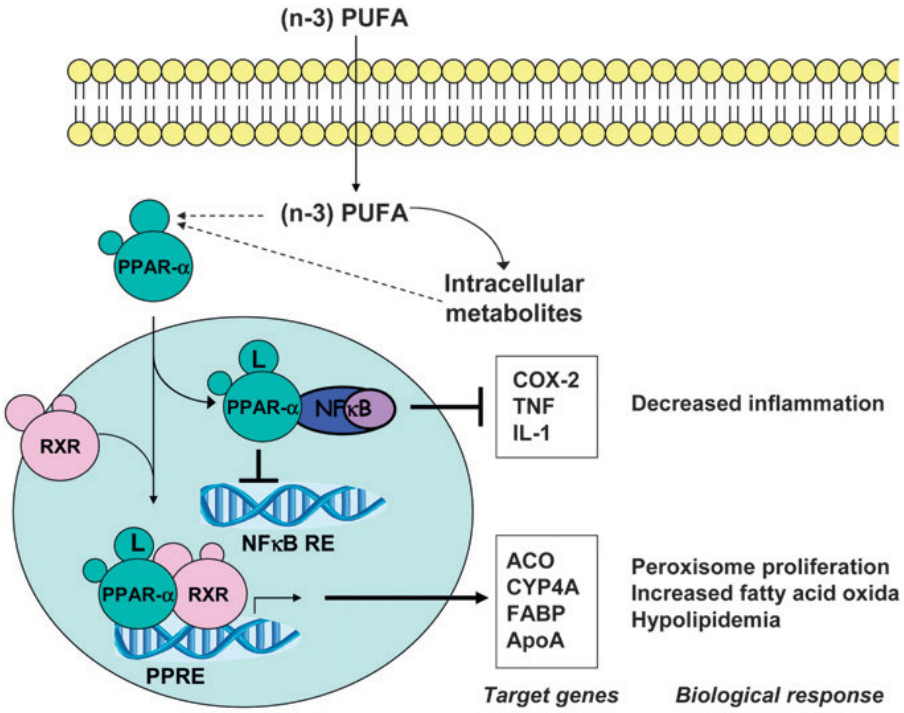



**Figure 1.13. The PPARα pathway.** Through activation of PPAR, omega-3 (n-3) PUFAs are able to regulate metabolism and other cell and tissue responses, including adipocyte differentiation and inflammation. **Abbreviations:** ACO= acyl CoA oxidase; ApoA= apolipoprotein A; COX= cyclooxygenase; CYP4A= cytochrome P450 4A; FABP= fatty acid binding protein; L= ligand; NFkB= nuclear factor kappa B; PPAR= peroxisome proliferator-activated receptors; RXR= retinoic acid receptor. Source: Calder, 2012. *J Nutr* **142**(3): 592-599.

### 1.1.21.9 Advantages over Other Therapies

As the current treatments to tackle obesity are lacking, the discovery that omega-3 PUFAs have such positive effects makes for an interesting avenue of research. As many individuals are reluctant to take pharmaceuticals, and compliance issues are a problem with the use of energy-restrictive diets and physical activity, the simple suggestion to introduce omega-3 PUFAs to an individual's diet may meet with greater compliance. The effects of nutrients on body weight are discussed in more detail in the following sections.

## 1.2 Importance of Diet Composition in Body Weight Regulation

### 1.2.1 Macronutrient Effects on Body Weight

The major groups of nutrients have been classified according to the amounts in which they are required, their chemical nature and functions in the body. There are two types of nutrients, macronutrients and micronutrients. Macronutrients are defined as the components within a diet that provide sources of energy to the individual, of which there are three groups: protein, fat and carbohydrate. In comparison, micronutrients are the nutrients required in small quantities, to control a range of physiological functions but which the individual cannot produce themselves. These include minerals and vitamins which, although essential, do not have an energy component to them (Barasi, 2007).

### 1.2.2 Carbohydrates

A carbohydrate is an organic compound comprised of carbon, hydrogen and oxygen molecules. Carbohydrates are saccharides, easily divisible into four groups: monosaccharides, disaccharides, oligosaccharides, and polysaccharides. These groups vary in degree of complexity. The monosaccharides and disaccharides form simple carbohydrates (refined sugars) (Flitsch & Ulijn, 2003), whereas the oligosaccharides and polysaccharides are more complex (Barasi, 2007). Monosaccharides are the simplest type of carbohydrate; examples include glucose, fructose, lactose and ribose. Monosaccharides are the precursors for the disaccharides, such as sucrose, and polysaccharides such as starch. Polysaccharides function as energy stores (e.g. starch and glycogen) and as structural component of cells (e.g. cellulose and chitin). Oligosaccharides contain a small number of simple sugars and often remain undigested until they reach the colon where they undergo fermentation serving as a food source for intestinal microflora (Barasi, 2007).

Saccharides and their associated derivatives include many important biological molecules such as the monosaccharide ribose, which is a component of RNA, and deoxyribose, a component of DNA (Flitsch & Ulijn, 2003). Some carbohydrates resist digestion and comprise the non-starch polysaccharides which are part of the dietary fibre which aids in the function of the gastrointestinal tract (Barasi, 2007). In terms of food consumption, complex carbohydrates such as

cereals, bread, and pasta contain starch, whereas the simple carbohydrates, found in sweets and jams, contain refined sugars.

#### 1.2.2.1 Carbohydrate Metabolism

The most utilised of the carbohydrates is the monosaccharide glucose, which can be successfully metabolised by all organisms. Simple carbohydrates, such as refined sugars, can be broken down in cells. More complex carbohydrates, such as the disaccharide sucrose, are broken down in the small intestine by enzymes specific to the sugar which split the chain releasing simple sugars. In the instance of sucrose, this is broken down by sucrase. If sucrase is not secreted, then intolerance will develop which can lead to malabsorption and diarrhoea. Another carbohydrate example is the polysaccharide starch, which is metabolised by the enzyme amylase, present in saliva of the mouth. This enzyme breaks the starch into amylose which is then further digested in the duodenum into maltose and glucose (Barasi, 2007).

There are other carbohydrates, such as cellulose, that humans cannot digest as they do not produce the enzymes necessary for the process. Carbohydrates are an instant fuel source because they are easier to metabolise than fats and protein. For all mammals, glucose is the most important carbohydrate, and the brain is fuelled primarily by this sugar. The concentration of circulating plasma glucose is involved in the control of insulin concentrations (Barasi, 2007).

#### 1.2.2.2 Glucose Regulation

When carbohydrates are metabolised, glucose is transported from the small intestine across the apical membrane and into the bloodstream by a twostep process. Firstly, glucose transporter proteins, present in cell membranes, facilitate the movement of glucose down its concentration gradient from the lumen of the small intestine into the apical cells. Secondly, glucose moves from the apical cells into the bloodstream via facilitated diffusion (Barasi, 2007). This process allows for the maintenance of steady concentrations of glucose in the body, but this can also be helped by choosing foods with certain glycaemic index. The glycaemic index provides an indication of how blood glucose concentrations change after the consumption of different carbohydrates. Essentially, glycaemic index is a measure of how quickly food glucose is absorbed. Lower glycaemic index foods are preferable in situations where

an extended period of glucose release is necessary, for example to regulate diabetic control, or for athletes in training for sporting events (Barasi, 2007).

#### 1.2.2.3 Dietary Treatment of Type 2 Diabetes

As mentioned previously, type 2 diabetes is a disease increasing in prevalence due to the rise in the number of obese individuals. This disease results from a dysfunction in carbohydrate metabolism as characterised by hyperglycemia (McGeoch *et al.,* 2011). The importance of glycaemic control in the prevention of chronic complications associated with diabetes was demonstrated by the UK Prospective Diabetes Study (Stratton *et al*., 2000). The consumption of carbohydrates is one of the main factors affecting glycaemic control and both the quantity, and quality (as indicated by the glycaemic index), of the ingested carbohydrate has an effect on diabetic control (Jenkins *et al.,* 1981). The regular consumption of foods with a high glycaemic index has been linked with the development of several conditions such as diabetes, obesity and cardiovascular disease, whereas low glycaemic index foods are recommended as preventative measures and even treatments (McGeoch *et al.,* 2011). These diets, low in glycaemic index, tend to have a higher amount of fibre which aids in the absorption of dietary cholesterol, contributing to a greater release of satiety signals and reducing the likelihood of feeling hungry, thus helping to maintain body weight control. In patients with type 2 diabetes, this results in a lower insulin discharge which helps to maintain a normalised, steady, blood glucose concentration (Jenkins *et al.,* 2002 Willett *et al.,* 2002; Colombani, 2004). Additional macronutrients which effect body weight regulation are described in the following sections.

### 1.2.3 Fatty Acids

The consumption and composition of dietary fatty acids has been the subject of extensive research because of the involvement of fats in the development of diseases such as obesity, diabetes and cardiovascular disease. Fats are essential in the diet to provide a source of energy, structural components within the body and as a constituent of metabolic pathways. Most dietary fats take the form of triglycerides (TGs), which contain three fatty acids (FAs) attached to a glycerol backbone. These fatty acids are the main components of dietary lipids. Their basic structure comprises a carbon backbone, with a carboxyl (-COOH) group at one end and a methyl group (-

CH3) at the other end. The FAs differ from one another in their chain length and number of double bonds (degree of saturation). These variations determine how they are metabolised and therefore, their effects on health. Specific FAs are important for cell membrane structure and function. There are several general categories of these fatty acids; these include the saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) (Moussavi *et al.,* 2008).

A FA nomenclature system exists as follows: number of carbon atoms, number of double bonds, followed by location of first double bond (carbon atom counting from the methyl group end); e.g. 18:1 (n-9) (oleic acid) is an 18-carbon chain with one double bond located at the 9th carbon from the CH3 end; n is sometimes written as omega (ω); e.g. n-3 = ω-3. Additional examples can be seen in Table 1.1.

### 1.2.4 Saturated Fatty Acids

The SFAs contain no double bonds along the chain length and have the maximum number of hydrogen atoms on each carbon molecule, and because of this, they tend to be solid at room temperature. Milk fat, containing SFAs synthesised by bacteria in the bovine rumen, is the main dietary source of short-medium chain SFAs (4-10 carbons), and these are present in butter products. The majority of dietary SFAs are medium chain (14-18 carbons) and come from palm oil, coconut oil, animal fats and hydrogenated fats. Longer chain SFAs (24 carbons) are synthesised within brain tissue membranes (Barasi, 2007). An example of a SFA can be seen in Figure 1.14 (also see Table 1.1).

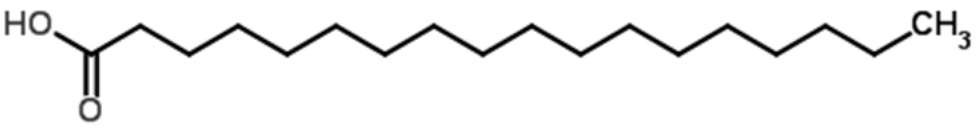



**Figure 1.14. Chemical structure of the SFA, stearic acid**. Stearic acid has 18 carbon molecules and is present in many animal fats. However, it is also present in high concentrations in cocoa and shea butter. **Abbreviations:** C= carbon; H= hydrogen; O= oxygen. Source: www.chemspider.com

SFAs are one of the main culprits behind the current obesity epidemic. One of the likely reasons behind this is their role in passive overconsumption.

### 1.2.5 Role of Passive Overconsumption in Obesity

When eating, food items provide sensory signals to the body through taste, texture, smell, sight, and sound. These signals then prompt a postingestive response within the body (Stubbs *et al.,* 2001; Brunstrom, 2007). Because of this, an individual learns to expect certain satiety responses based on the sensory signals of the foods consumed. However, in today's food climate, the sensory signals are often disconnected from their original nutritional consequence. For example, when an individual consumes food items such as cake, which is high in fat content, they often have difficulty identifying these foods as actually being high in fat (Drewnowski, 1988; Drewnowski & Schwartz, 1990; Lawton *et al*., 2000; Stubbs *et al.,* 2001). This is because the item lacks the sensory properties associated with fat, such as thick but creamy texture, and therefore, one is unable to recognise the real nutrient content of the item due to inappropriate sensory signalling (Drewnowski, 1995). This disconnection between sensory and metabolic messages results in an inadequate adjustment of energy intake when consuming food items which are high in fat and energy dense (Blundell & MacDiarmid, 1997; Mazlan *et al.,* 2006; Westerterp, 2006). The consumption of these types of food then stimulates passive overconsumption, which ultimately leads to weight gain (Viskaal-van Dongen *et al.,* 2009). Studies have shown that following episodes of overconsumption, in many instances, individuals do not adjust their energy intake to return to an energy balance situation (Levitsky *et al.,* 2005; Jebb *et al.,* 2006; Viskaal-van Dongen *et al.,* 2009). These results support an inability to detect a high energy intake, which may result from sensory signals that are not in accordance with the actual nutrient content of the food (Viskaal-van Dongen *et al.,* 2009).

Furthermore, the passive overconsumption effect of eating a diet high in fat is likely linked to the actual high energy density of high-fat foods (Blundell & MacDiarmid, 1997). Dietary fat provides double the amount of energy per gram of food intake than either protein or carbohydrates (Poppitt & Prentice, 1996); therefore, a meal consisting of high-fat food is usually smaller in weight than a high carbohydrate meal leading the individual to eat again sooner (Castellanos & Rolls, 1997). This may also be compounded by the fact that diets high in fat exert a weaker effect on satiety than protein and carbohydrate (Blundell *et al*., 1993; Blundell & MacDiarmid, 1997; Fernández-Quintela *et al*., 2007). The process of passive

overconsumption is thought to play a major role in the development of obesity; however, not all FA types influence consumption in this way, in fact, some have opposing effects.

### 1.2.6 Monounsaturated Fatty Acids

In comparison to SFAs, MUFAs contain one double bond. An example of this is oleic acid (see Figure 1.15 and Table 1.1), derived primarily from rapeseed and olive oil. Fish oil may also provide a small amount of MUFAs (Barasi, 2007).

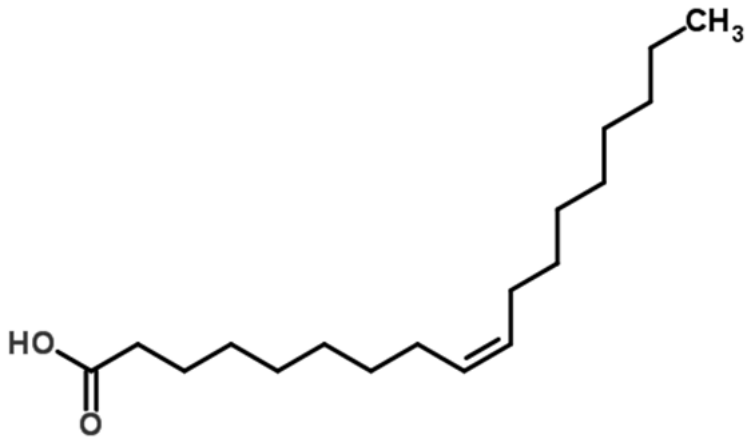



**Figure 1.15. Chemical structure of the MUFA, oleic acid**. Oleic acid has 18 carbon molecules and one double bond. It is present in olive and rapeseed oil, as well as some animal fats. **Abbreviations:** C= carbon; H= hydrogen; O= oxygen. Source: www.chemspider.com

### 1.2.7 Polyunsaturated Fatty Acids

Finally, PUFAs have the lowest melting point of all the FAs because of the multiple double bonds in their structure, and they form a liquid at room temperature. The majority of PUFAs contain 18-22 carbon molecules and up to six double bonds; these belong to the omega-6 and omega-3 families (see Figure 1.16, 1.17 and Table 1.1). The families are dictated by the position of the first carbon-carbon double bond. PUFAs regulate the fluidity and other properties of cell membranes, with particular roles in the brain, CNS and retina. They are also precursors of the eicosanoids, which are involved in the regulation of metabolic processes. Eicosanoids from the omega-3 and omega-6 families vary in their potency and function, and an altered balance can affect functions such as inflammation. The body is unable to synthesise the omega-3

FAs; DHA and EPA, and therefore, they must be supplied from dietary sources (Barasi, 2007).

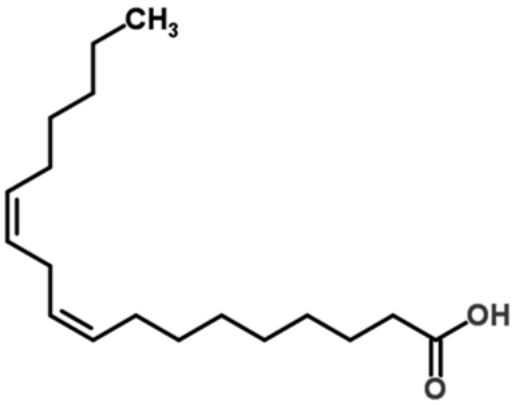
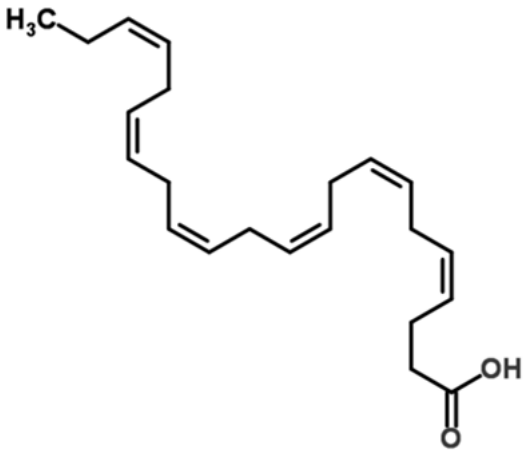



**Figure 1.16. Chemical structure of the omega-6 PUFA, linoleic acid**. Linoleic acid has 18 carbon molecules and two double bonds. It is present in vegetable oils such as sunflower oil. **Abbreviations**: C= carbon; H= hydrogen; O= oxygen. Source: www.chemspider.com

**DHA**




**EPA**

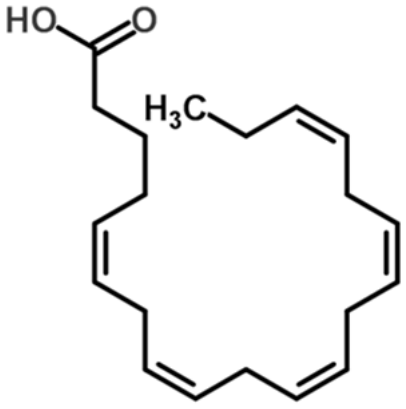



**Figure 1.17. Chemical structures of the omega-3 PUFAs, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)**. DHA has 22 carbon molecules and six double bonds, whereas EPA has 20 carbon molecules and 5 double bonds. Both DHA and EPA are present in fish oil. The body cannot generate these PUFAs, and therefore, they must be consumed in the diet. Abbreviations: C= carbon; H= hydrogen; O= oxygen. Source: www.chemspider.com

Expansion of the nomenclature system for the example FAs included above can be found in Table 1.1.

**Table 1.1. Nomenclature for example FAs.** The common name, nomenclature and chemical name for all FA examples included here.

| Common Name | Nomenclature | Systematic Name |
|---|---|---|
| Stearic acid | 18:0 | Octadecanoic acid |
| Oleic acid | 18:1 (n-9) | (9Z)-9-Octadecenoic acid |
| Linoleic acid | 18:2 (n-6) | (9Z,12Z)-9,12-Octadecadienoic acid |
| Docosahexaenoic acid | 22:6 (n-3) | (4Z,7Z,10Z,13Z,16Z,19Z)-4,7,10,13,16,19-Docosahexaenoic acid |
| Eicosapentaenoic acid | 20:5 (n-3) | (5Z,8Z,11Z,14Z,17Z)-5,8,11,14,17-Icosapentaenoic acid |

### 1.2.8 Properties and Metabolism of Fatty Acids

Saturated and unsaturated fats vary in their energy content and melting point. As unsaturated fats (MUFAs and PUFAs) contain fewer carbon-hydrogen bonds than SFAs with the same number of carbon atoms, they will yield slightly less energy during metabolism than SFAs with the same number of carbon atoms (see Figures 1.14-1.17). SFAs can coordinate themselves into a closely packed arrangement, so they can freeze easily and are typically solid at room temperature (see Figure 1.18). For example, animal fats, such as lard, are high in SFA content and are solid in form. In comparison, MUFAs and PUFAs, which often come from olive and fish oils, are highly unsaturated and form an oily liquid at room temperature (Moussavi, 2008).

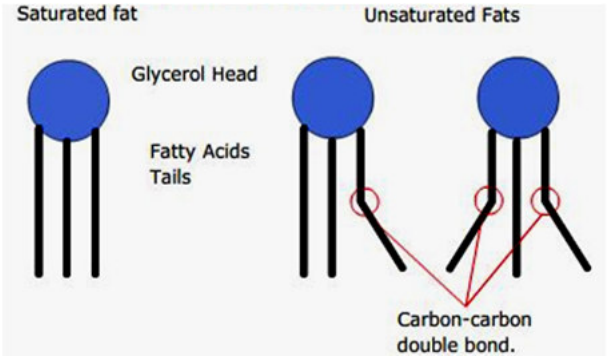



**Figure 1.18. Diagrammatic representation of fatty acid types.** Fatty acids contain a glycerol head with a fatty acid tail. The unsaturated (MUFA and PUFA) examples contain carbon-carbon double bonds which appear as kinks in the FA tail. In comparison, the SFA has a straight tail which allows them to be closely packed together. Source: Adapted from www.sciencedirect.com.

The variability in fatty acid structure, such as chain length, degree of unsaturation, and position of the double bonds affects the rate at which the fatty acid is oxidised (DeLany *et al.,* 2000; Moussavi, 2008). Carbon labelling techniques have shown that PUFAs oxidise more rapidly than SFAs (Mead *et al.,* 1956; Cenedella & Allen, 1969). Measurement of FA oxidation in rats with a range of fatty acids has shown that oxidation of SFAs decreases with increasing carbon chain length. However, for unsaturated fatty acids, the medium chains FAs (8–14 carbons) are oxidized the most rapidly (Leyton *et al.,* 1987). More recent human studies have shown that oxidation of oleic acid (MUFA) is greater than that of linoleic acid (omega-6 PUFA), which is greater than that of stearic acid (SFA). Therefore, short- and medium-chain unsaturated fatty acids (oleic and linoleic acid) are oxidised more rapidly than the long chain SFAs (palmitic and stearic acid) (DeLany *et al.,* 2000). Human feeding studies, in which the ratios of PUFA to SFAs in the diet are altered, suggest that PUFAs are more greatly oxidised than the SFAs in general (Jones & Schoelloer, 1988). These results confirm the findings of others that the PUFAs, linoleic acid (omega-6) and linolenic acid (omega-3), are highly oxidised in comparison to SFAs (DeLany *et al.* 2000). The observed differences in rates of oxidation may also relate to differences in weight gain observed when different types of FA are consumed. As PUFAs are more highly oxidised than the SFAs, this may lead to less storage of the PUFAs, in adipose depots, in comparison to the SFAs, hence these animals gain less weight (Mercer & Trayhurn, 1984; Cunnane *et al.,* 1986; Jones, 1989; DeLany *et al.,* 2000).

### 1.2.9 Differential Effects on Body Weight and Adiposity

It is well established that those who eat a diet high in fat are more likely to become obese than those eating a diet low in fat (Blundell & King, 1996; Blundell & MacDiarmid, 1997). This is evident in both humans and rodents, as shown in studies where the intake of a high-fat diet (HFD) can cause severe obesity, even in the absence of increased energy intake (Oscai *et al*., 1984). The effects of HFDs on body weight depend not only on the amount consumed, but also on their energy content and how they are metabolised. As triglycerides, which are not immediately used for fuel, are stored in adipose tissue (Barasi, 2007), a diet high in fat will encourage weight gain, but how much adipose tissue accrues, and where it is deposited, will depend on whether the fats are SFAs or PUFAs (Fernández-Quintela *et al.*, 2007).

The results of some short-term studies indicate that PUFAs exert a relatively stronger control over appetite than SFAs and this is likely to be due to mechanisms involving the control of the gut satiety hormone CCK and the varying oxidative capacity of ingested fats (Lawton *et al*., 2000; Moussavi *et al*., 2008). When the receptors in the gut lining detect the presence of fat in the intestine, CCK is secreted by I-cells in the mucosal epithelium of the small intestine, slowing down the pace of digestion by inhibiting gastric emptying and gastric acid secretion (Dockray, 2004; Cummings & Overduin, 2007). This, along with raised enzymatic secretions from the pancreas, and increased hepatic bile into the intestine, allows for the complete digestion of fats by catalytic reactions. Additionally salts present in the bile cause emulsification of the fats, therefore, aiding in digestion and absorption (Capasso & Izzo, 2008). As the levels of fat drop, the concentration of CCK also reduces. Circulating concentrations of CCK increase when fed a diet high in PUFAs in comparison to one high in SFAs which may explain their differential effects on satiety (Beardshall *et al*. 1989; Relling & Reynolds, 2007). As CCK is also a neuropeptide, it works as a satiety factor by acting on the corresponding receptors in the CNS (see Figure 1.7; Morton *et al.,* 2006; Dockray, 2009). The mechanism behind the suppression of hunger is thought to be due to the decrease in gastric emptying (Shillabeer & Davison, 1987), and the satiety effects of CCK are reduced in obese rats (Fink *et al.,* 1998).

### 1.2.10 Protective Effects of PUFAs on Body Weight

Feeding rats diets high in the omega-3 PUFAs, EPA and DHA, found in fish oils has been shown to improve protection against body fat gain (Belzung *et al.,* 1993; Hainault *et al.,* 1993; Hill *et al.,* 1993; Baillie *et al*., 1999). This is important, as the incidences of glucose intolerance, which precedes obesity-induced type 2 diabetes in humans, is affected by both the amount and distribution of body fat (Kissebah *et al*., 1982).

Studies using rodent models have shown that PUFAs attenuate the accumulation of adipose tissue resulting from consumption of SFAs (Buckley & Howe, 2009). They do this by inhibiting expression of lipogenic genes and stimulating transcription of those involved in lipid oxidation (Fernández-Quintella, 2007). Additionally, when SFAs are incorporated into the membranes of cells they have the capacity to reduce metabolic rate and reduce β3 adrenoceptor binding

(Matsuo & Suzuki, 1997). This has implications for energy balance, as the β3 adrenoceptor is present in adipose tissue and aids in the enhancement of lipolysis (Ferrer-Lorente *et al.,* 2005). Conversely, the addition of PUFAs to the diet increases membrane fluidity (Fernández-Quintella, 2007) and β3 adrenoceptor affinity (Nicolas *et al*., 1991). In addition to their roles as energy and cell-structural elements, it is now clear that fatty acids act as potent gene regulators on enzymes of endogenous lipid synthesis and adipocyte proliferation (Clarke *et al*. 1997; Storlien *et al*., 2000).

Therefore, to summarise, SFAs, found mostly in animal fats (e.g. in meat) and dairy products, tend to be less satiating, and increase body weight by increasing adiposity. In contrast, PUFAs may enhance satiety and promote weight loss. These include the essential fatty acids, which must be supplied in the diet; i.e. the omega-3 PUFAs (e.g. in fish oils) and omega-6 PUFAs (e.g. in seed oils). However, due to discrepancies in the results of some studies, additional work is required to create a rational rat model of chronic PUFA consumption (see Chapter 2 for more detail). The use of isocaloric diets will remove the confounding effects of varying energy contents, allowing for the direct comparison of different macronutrients. However, macronutrients should ideally be tested in isolation as many of the dietary studies involving the consumption of an HFD have also included sucrose, which is known to promote insulin resistance (Kokoeva *et al.,* 2005; Chang *et al.,* 2008; Hwang *et al.,* 2008; Hofmann & Tschöp, 2009; Kim *et al.,* 2009; Park *et al.,* 2010; Rivera *et al.,* 2011; Boitard *et al.,* 2012; Lee *et al.,* 2012; McNay *et al.,* 2012; Thaler *et al.,* 2012; Yoo *et al.,* 2012).

Currently, of the environmental factors which impact on body weight, nutrients are the most influential. Within normal limits, hypothalamic and related neuronal populations correct perturbations in energy metabolism, to return the body to its nutritional set-point, either through direct response to nutrients, or indirectly via peripheral appetite signals. Excessive intake of certain macronutrients, such as simple carbohydrates and SFAs, can lead to obesity and attendant metabolic dysfunction, also reflected in alterations in structural plasticity, and intriguingly, neurogenesis, in some of these brain regions. Some of these alterations are discussed in more detail in the following sections of this review.

## 1.3 Role of Neuroplasticity in Body Weight Regulation

Neuroplasticity refers to changes in neural pathways which are due to changes in behaviour, environmental influences, neural processes and occasionally bodily injury (see Figure 1.19; Pascual-Leone *et al.,* 2011). Neuroplastic changes in the hypothalamus, including altered neurochemical phenotype, neuronal activation, synaptic connections, and dendritic growth and pruning, can be stimulated by dietary factors, not only during critical periods of development, but also in adulthood (Wang *et al.,* 1999; LaBelle *et al.,* 2009; Bouret, 2010; Horvath *et al.,* 2010; Ravussin *et al.,* 2011; Lee *et al.,* 2012; McNay *et al.,* 2012). This is also true of neurogenesis (Chang *et al.,* 2008; Kokoeva *et al.,* 2005; McNay *et al.,* 2012), the process by which neurons are born, proliferate, differentiate and integrate into established circuitry (Eriksson *et al.,* 1998; Balu & Lucki, 2009). The technical aspects of measuring neurogenesis are described in Chapter 2.

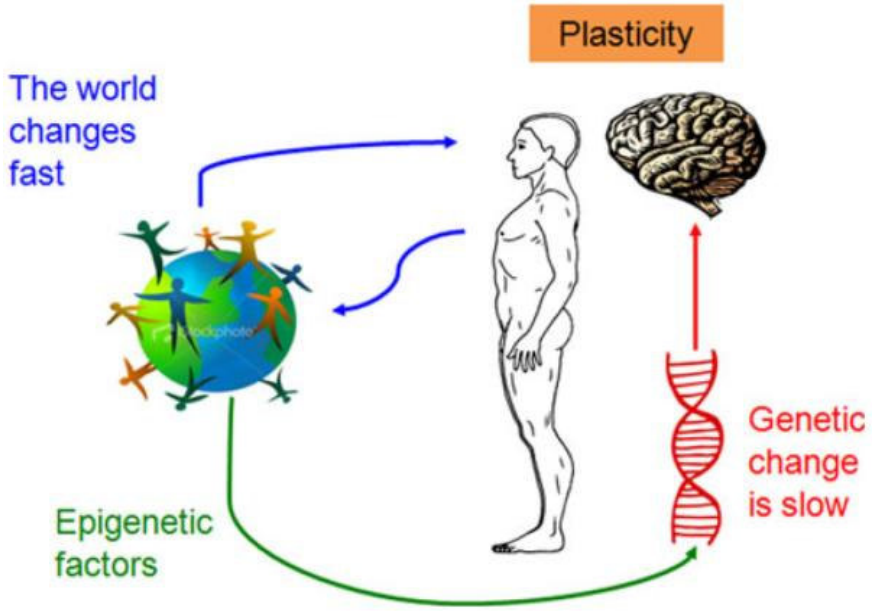



**Figure 1.19. Schematic representation of the concept of plasticity.** Brain plasticity allows for rapid adaptation to environmental changes that occur quicker than genetic or epigenetic response times. Source: Pascual-Leone *et al.,* 2011. *Brain Topogr* **24**(3-4): 302-315.

### 1.3.1 Neuroplastic Changes in Response to Diet

In the hypothalamus, cells which line the 3V have stem cell capabilities in the adult and can proliferate into neurons which migrate to nearby nuclei (Kokoeva *et al.,* 2007) and are engaged in responding homeostatically to peripheral satiety signals,

such as leptin (Zivlan *et al*., 2009). Expression and release of such signals is determined, not only by volume of food intake, but also diet composition, as demonstrated by the consumption of diets high in SFAs and PUFAs which have roughly opposing effects on energy metabolism. For instance, SFAs alter appetite to drive overeating, which is expressed as disturbed feeding behaviour (meal patterns) (Lawton *et al*., 2000; Wang *et al.,* 2002), and consequently, to induce weight gain. They do this by overriding the effects of satiety hormones (Lawton *et al*., 2000) and reducing the expression of appetite-stimulating neuropeptides within hypothalamic nuclei (Wang *et al*., 2002). In addition, high-SFA intake impairs the growth of new neurons (neurogenesis) in the adult brain. This occurs even before the onset of obesity (Park *et al.,* 2010; Kanoski & Davidson, 2011). In contrast, PUFAs attenuate weight gain, but what mediates this attenuation centrally is unclear. As dietary restriction is known to induce both hypothalamic neurogenesis (Lee *et al.,* 2002) and weight loss in rodent models (Wang *et al.,* 2002), it is possible that PUFAs control body weight by mimicking effects of dietary restriction.

### 1.3.2 Development of Obesity

Developmental exposure of animals or humans to a range of nutritional and metabolic insults, such as maternal obesity, diabetes, malnutrition, and early postnatal over-nutrition can result in predisposition to obesity in later life (Levin, 2000; Plagemann, 2006; Gillman *et al.,* 2008). Both maternal obesity and gestational diabetes increase the risk of obesity and type 2 diabetes in the offspring (Levin & Govek, 1998; Taylor & Poston, 2007). Furthermore, malnutrition in the mother also increases the likelihood of the offspring developing obesity in later life (Field *et al.,* 2005; McMillen *et al.,* 2005).

### 1.3.3 Critical Periods

The developmental programming of brain feeding pathways *in utero* represents a potential underlying cause for obesity development (Horvath & Bruning, 2006; Bouret, 2009). Sensitive periods of brain development, during which the brain is sensitive to environmental exposures, occur due to the processes of cell proliferation, migration, differentiation, axon growth and apoptosis (Bouret, 2010). The first important phase for hypothalamic development is formation, with neurogenesis occurring in the prolific ependymal lining of the 3V, during mid-gestation in rodents.

Migration and differentiation occur during late embryonic development (Markakis, 2002). The interruption of any of these stages can result in structural and functional abnormalities of the hypothalamus (Davis *et al.,* 2004). The second phase of hypothalamic development is during the early weeks of postnatal life, when neurons send out axonal projections to their specific target sites. Studies have shown that ARC projections are immature at birth but develop at week two of postnatal life (Grove *et al.* 2003; Bouret *et al.,* 2004). Different regions of the hypothalamus develop at different times, and the projections from the DMH and VMH develop before those in the ARC. Hypothalamic development in rodents can take days, whereas in humans, it can take weeks or months. Also, rodents undergo more postnatal development than humans, who undergo more prenatal maturation of hypothalamic structures (Bouret *et al.,* 2004).

Hormones have been shown to play an important role in hypothalamic development (Simerly, 2002). Leptin deficiency in mice results in abnormal development (stunted growth), and reduced number, of axonal projections from the ARC to other regions of the hypothalamus. During early postnatal life, leptin acts as a trophic signal, influencing developmental stages in pathways that will aid leptin signalling in mature mice (Bouret *et al.,* 2004). However, leptin can still affect the brain in adults, resulting in the rearrangement of excitatory and inhibitory inputs on ARC neurons, suggesting that this particular brain circuit remains relatively plastic throughout adult life (Pinto *et al.,* 2004). Other hormones such as insulin have also been shown to have a role in the developing hypothalamus, as demonstrated by injection of postnatal insulin, resulting in morphological changes in the VMH (Plagemann *et al.,* 1999). Furthermore, mature hypothalamic neurons are also sensitive to external influences. For example, an injection of ciliary neurotrophic factor (CNTF) in adult obese mice induces increased neurogenesis in the hypothalamus (Kokoeva *et al.,* 2005). These results support the ideas that hypothalamic plasticity remains sensitive to neurotrophic cues during adult life. However, the extent and type of response may differ between adults and neonates (Bouret, 2010).

### 1.3.4 Influence of Maternal Nutrition on Obesity

As mentioned previously, the hypothalamus is exposed to two major environments; the first *in utero*, and the second postnatally. These time periods represent important phases during which the alteration of metabolic and hormonal signals may result in abnormal hypothalamic development (Bouret, 2010). Studies have shown that with animal models of maternal obesity, prenatal nutrition influences the development of hypothalamic appetite-regulating networks. HFD feeding during pregnancy and lactation results in neonates with hyperphagia and obesity, associated with dysregulation of hypothalamic gene expression (Kirk *et al.,* 2009; Morris & Chen, 2009), and abnormal central leptin sensitivity in the ARC. This reduces the number and length of neural projections from the ARC to the PVN (Kirk *et al.,* 2009). These studies imply that the correct function of leptin during neonatal life is crucial for the maintenance of energy homeostasis and hypothalamic function (Bouret, 2010).

These types of changes in neuroplasticity can be influenced by both genetics and the environment (dietary influence). Studies have shown that by exposing late fetal and neonatal rats to increased HFD *via* feeding of the mother, the neonatal rat displays several of the physiological and behavioural changes linked to obesity, including elevated concentrations of leptin, increased body weight gain and food intake, modest preference for fat-containing food types, as well as the onset of hypothalamic leptin resistance. In contrast, in the hippocampus, both the fetal and neonatal rodent treated with leptin show similar increases in neurogenesis and survival of newborn neurons (Walker *et al.,* 2008). Alterations of neurochemical phenotype which relate to obesity also occur in the adult, as discussed below.

### 1.3.5 Adult Obesity

Dietary macronutrients differentially affect both brain structure and activity at the cellular level. Particularly striking results have been observed in rodent models, when comparing the effects of diets enriched with different types of FAs. Work by Levin and colleagues has shown that these plastic changes also appear to underpin susceptibility and resistance to obesity. For example, they showed that rats with diet-induced obesity (DIO) from consumption of an HFD (31% of energy as fat), had long primary dendrites on VMH neurons which were 19% shorter than those in their diet-resistant (DR) counterparts (La Belle *et al.,* 2009). The work of Storlien and

colleagues compared mice fed HFDs containing 58% of energy as fat from different sources (SFAs, omega-3 PUFAs, or omega-6 PUFAs). They showed that c-Fos immunoreactivity (where c-Fos is an early gene marker of neuronal activation) was increased in the LHA (appetite centre) and decreased in the VMH (satiety centre), following one week of SFA consumption (Wang *et al.,* 1999). In contrast, both omega-3 and omega-6 PUFA-fed mice showed unchanged activation in the LHA, but an increase in the VMH. At seven weeks, following substantial fat accumulation, the SFA-fed mice demonstrated an increase in PVN activity; however, following a switch to the omega-3 PUFA diet for four weeks, this activation was reversed by approximately 50%, and activation in the ARC was increased by 400%. These results demonstrated that dietary SFAs influence hypothalamic neuronal activity by increasing levels of activation in the lateral regions, but by decreasing levels in the ventromedial regions, as obesogenic effects develop. In contrast, omega-3 PUFA diets reverse this effect. The mechanisms behind these relationships were unexplained; however, it was suggested that the direct effects of FAs on ARC leptin receptor activation and downstream signalling may be involved (Wang *et al.,* 1999). Indeed, the cellular machinery (transporters, receptors and metabolic pathways) required for direct sensing of nutrients, including FAs and glucose, are known to exist in the neurons of the discrete hypothalamic regions involved in feeding regulation (Levin, 2002; Lam *et al.,* 2005; Levin *et al.,* 2006).

### 1.3.6 Neurogenesis in the Adult Brain

More recently, research attention has been turning to the role of neurogenesis in the regulation of body weight, and the differential effects of dietary macronutrients on this process are only beginning to emerge.

Neurogenesis is the process by which neurons are born, proliferate, differentiate, migrate and then integrate into already established brain circuitry. These stages are regulated by a diverse range of factors present in the neurogenic niche, including intrinsic (e.g. transcription factors) and extrinsic molecular signals (trophic factors), as well as neurotransmitters (dopamine and amino acids; Pathania *et al.,* 2010).

Previously thought to occur only in the embryo, neurogenesis has now been demonstrated in the adult brain (Eriksson *et al.,* 1998; Balu & Lucki, 2009) and can

be influenced by a range of factors, including age, sex, stress, environmental enrichment and animal species and strain (Balu & Lucki, 2009; Bonfanti & Peretto, 2011; Clark *et al.,* 2011; Ming & Song, 2011; Simpson & Kelly, 2011; Kageyama *et al.,* 2012).

### 1.3.7 Hippocampal Neurogenesis

The most prolific neurogenic niches have been identified as the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus (DG), within the hippocampus (see Figure 1.20; Balu & Lucki, 2009). The hippocampus is crucially involved in the regulation of cognition and mood. A prevailing assumption in the field of adult neurogenesis is that the continuous generation of new neurons within the hippocampus offers, in addition to synaptic plasticity, a cellular flexibility to this system (Couillard-Despres *et al.,* 2011), and that this supplementary population is especially important for memory and learning. Indeed, newly generated neurons are particularly excitable and can therefore be more sensitive to incoming input (Schmidt-Hieber *et al.,* 2004; Couillard-Despres *et al.,* 2006). Many studies support the concept that young neurons contribute to and facilitate the learning of hippocampal-dependent tasks (e.g. Gould *et al.,* 1999; Deng *et al.,* 2009; Tronel *et al.,* 2010; Couillard-Despres *et al.,* 2011). This assistance could be mediated, for example, through the lower threshold required for the induction of long-term potentiation in newly generated neurons (Ge *et al.,* 2007). Studies have shown that newly generated neurons are integrated very rapidly into the hippocampal network, and that they can trigger action potentials upon stimuli that are much weaker than those required to stimulate mature granule cells (Schmidt-Hieber *et al.,* 2004; Couillard-Despres *et al.,* 2006, 2011). Hence, the presence of numerous immature neurons in the hippocampal network might provide a higher sensitivity to incoming inputs, thereby facilitating information processing (Couillard-Despres *et al.,* 2011).

It is also well established that several growth factors are involved in the mechanisms regulating adult hippocampal neurogenesis (Lee & Son, 2009). The involvement of growth factors in the mechanism underlying the effect of anti-depressant drugs on hippocampal neurogenesis and anti-depressive disorders has been much investigated. Adult hippocampal neurogenesis is positively affected by

chronic anti-depressant treatment (Malberg *et al.,* 2000), and neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin-3 are known to be implicated in adult neurogenesis and neuroplasticity. Among these, BDNF has been intensively studied and shown to be involved in learning and memory, and synaptic plasticity (Poo, 2001). Spatial learning and memory is defective in a BDNF-deficient animal model (Mu *et al.,* 1999), and overexpression of BDNF causes both anxiogenic and antidepressant behaviour (Govindarajan *et al.,* 2009). Because of the traditional association between neurogenesis and the hippocampus, this brain region has been the main focus of studies investigating links between neurogenesis and diet. These studies have included alterations in feeding regimes, calorie restriction and dietary nutrients (Mattson *et al.*, 2003; Stangl & Thuret, 2009; Park *et al*., 2011), which are reviewed below (Section 1.3.9). The assumption that the hippocampus may be involved in such a relationship is not unreasonable, as hippocampal function is required for normal feeding behaviour (Davidson *et al*., 2007). However, whereas there is substantial evidence to suggest the functional significance of hippocampal neurogenesis in cognition, this is less so with respect to energy metabolism. Relationships between hippocampal neurogenesis and diet have been documented as phenomena, without significant insight into underlying mechanisms. It is true that obesity is becoming increasingly linked with depressed mood, impaired cognition and neurodegenerative diseases, including Alzheimer's, which are underpinned by neuroplastic changes (Bruce-Keller *et al.,* 2009; Barnes & Yaffe, 2011; Farooqui *et al.,* 2012). Indeed, the link between obesity and brain neuropathology has been substantiated by showing that weight reduction improves cognition (Siervo *et al.,* 2011). Thus, it has been proposed that adult hippocampal neurogenesis may link energy metabolism and cognition in order to regulate body weight (Park & Lee, 2011), functioning as an "interface" between the two (Vaynman & Gomez-Pinilla, 2006). However, direct evidence is lacking.

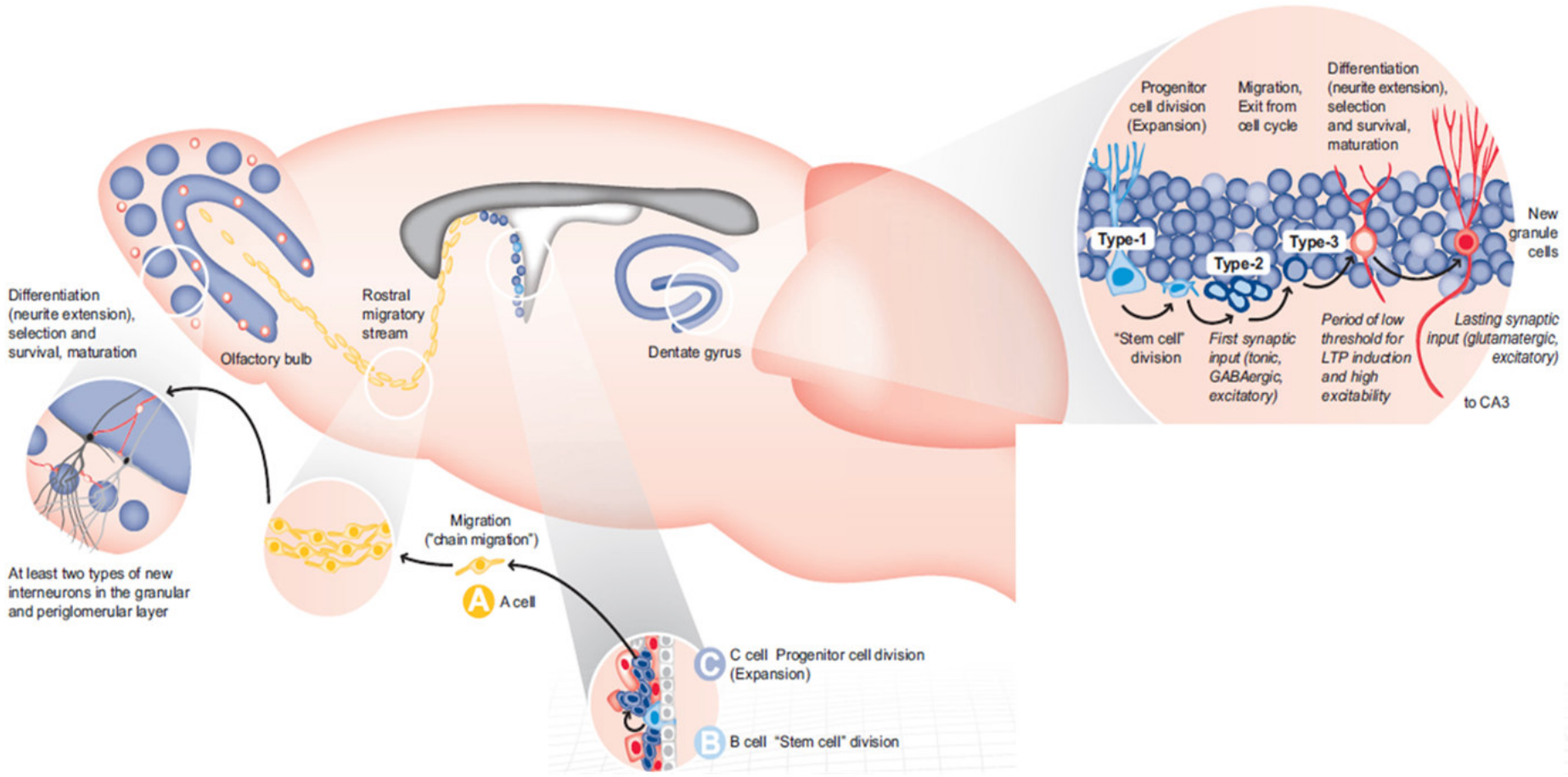



**Figure 1.20. Neurogenesis in the adult mammalian brain.** A sagittal section of rodent brain showing the sites of neurogenesis in the dentate gyrus (DG) of the hippocampus, and the olfactory bulb. Cells proliferate in the subgranular zone (SGZ) of the DG, from which they migrate and differentiate into mature neurons. Source: Kempermann *et al.,* 2004. *Trends Neurosci* **27**(8): 447-452. The hypothalamus is currently emerging as a neurogenic site, with cell-types A-C found in the wall of the third ventricle (Migaud *et al.*, 2010).

### 1.3.8 Role of Neurogenesis in Body Weight Regulation

#### 1.3.8.1 Hypothalamic Neurogenesis

Recently, the hypothalamus has also been shown to satisfy the criteria for a neurogenic niche in mammals (Magavi *et al*., 2000; Pencea *et al*., 2001; Migaud *et al.*, 2010; Yuan & Arias-Carrión, 2011). It has the capability of generating new neurons and expresses phenotypic markers of cell proliferation and neuronal fate, including the endogenous cell-cycle proteins, Ki-67 and proliferating cell nuclear antigen (PCNA), and uptake of the thymidine analogue, bromodeoxyuridine (BrdU). Newly formed cells within the 3V region migrate to appropriate areas of the parenchyma, including the LHA (Xu *et al*., 2005), where they then express a range of specific markers related to feeding metabolism (Markakis *et al*., 2004; Kokoeva *et al.,* 2007). More recent studies have also shown that newborn neurons are present in the mediobasal hypothalamus (Li *et al.,* 2012), as well as the tanycytes of the median eminence (Lee *et al.,* 2012). Hypothalamic neurogenesis is impaired by diet-induced and genetic forms of obesity, but can be restored by calorie restriction (McNay *et al.*, 2012). Until very recently, its function was thought to be simply to replace dead neurons (Markakis, 2002). As it is only recently that researchers have begun to identify the appetite-related factors which may mediate the relationship between diet composition, obesity and neurogenesis (in both hippocampal and hypothalamic sites), it is only now that the true extent of this functional role is finally emerging (Lindqvist *et al.,* 2006; Chang *et al.,* 2008; Park *et al.,* 2010; Rivera *et al.,* 2011; Boitard *et al.,* 2012; Lee *et al.,* 2012; Li *et al.,* 2012).

#### 1.3.8.2 Energy Restriction

Energy restriction, in the form of fasting, has been shown to increase hippocampal neurogenesis in rats and mice. Lee *et al.* (2000a, 2002b) showed that there were no differences in neurogenesis between energy-restricted and control animals at day one of fasting, but there were after three and four weeks of fasting. Studies from the same group suggested that energy restriction does not affect proliferation of neural stem cells, but does increase the survival of newly generated neural cells; and that improved learning and memory function shown in rats fed an energy restricted diet may be as a result of increased neurogenesis within the hippocampus (Mattson *et al.,* 2003).

Previously, it was assumed that all positive benefits associated with energy restriction occur as a result of a reduction in cumulative energy intake (Weindruch & Sohal, 1997). However, a more recent study showed that a fasting energy restriction paradigm induces weight loss in Sprague-Dawley rats but not in C57BL/6 mice. Measurement of energy intake showed that on days the mice had access to food they ate twice as much as their controls. However, the fasting mice did display anti-ageing changes equal to those maintained on a reduced energy diet, including reduced plasma insulin and glucose concentrations. Furthermore, fasting was more effective than daily restricted energy intake at protecting hippocampal neurons against injury (Anson *et al.,* 2003). As a result of the current obesity epidemic, and the difficulties in getting people to reduce their food intake, research into the development of pharmaceuticals, nutraceuticals and/or dietary restriction mimetics are crucial.

### 1.3.9 Effects of Dietary Macronutrients on Neurogenesis

As mentioned previously, one of the theories behind the sudden development of the obesity epidemic is the overconsumption of foods, specifically those with a high SFA and/or refined carbohydrate content. These macronutrients have been shown to cause changes in neuroplasticity, as described above, hence it is also possible that they influence neurogenesis.

#### 1.3.9.1 Simple Carbohydrates

The increase in the overconsumption of sugary foods and drinks is of major concern when addressing issues surrounding the increased prevalence of obesity. When considering high-sugar drinks, high-fructose corn syrup (HFCS) has been identified as an ingredient which may be detrimental (Purcell *et al.,* 2012; Stanhope, 2012). Well-known soft drink brands can contain from 48-65% HFCS (Ventura *et al.,* 2011; Sievenpiper *et al.,* 2012). Studies involving rodent models have elucidated a link between body weight gain and consumption of simple sugars in liquid format.

Rats consuming liquid sucrose or fructose (23% w/v) for four weeks showed no changes in body weight compared to controls consuming water, despite increased gonadal fat depots (van der Borght *et al.,* 2011). However, terminal serum concentrations of triglycerides, leptin, ghrelin and pro-inflammatory cytokines

(molecules which promote inflammation) were elevated in both groups, and newly mature neurons in the dentate gyrus were reduced by 50%, while numbers of apoptotic cells were increased. The authors suggested that elevated cytokines were responsible for the failure of neurons to survive, through both apoptosis and impairment of the BBB by triglycerides, which are known to compromise its integrity (Banks, 2008). Consequently, leptin is then prevented from crossing the BBB, depriving the hippocampus of its known neuroprotective effects (Kokoeva *et al.,* 2005; Garza *et al.,* 2008; Belsham *et al.,* 2009; Migaud *et al.,* 2010; Pierce & Xu, 2010; McNay *et al.,* 2012).

Interestingly, far lower concentrations of fructose (10%) have been associated with markers of metabolic syndrome, such as increased concentrations of circulating triglycerides, impaired glucose metabolism and increased abdominal adiposity, and in some cases, these changes have been observed in the absence of actual weight gain (Bocarsly *et al.,* 2010; Sheludiakova *et al.,* 2012). In contrast, additional studies have shown that six weeks of liquid carbohydrate diet containing 10% sucrose (w/v) had no effect on cell proliferation in the dorsal vagal complex (an additional region of the brain that has shown prolific activity), on body weight, or on circulating concentrations of BDNF (Zeeni *et al.,* 2009). BDNF is a growth factor which is involved in the control of body weight through central mechanisms, including neurogenesis (Mattson *et al.,* 2003; Vaynman & Gomez-Pinilla, 2006; Cordeira & Rios, 2011; Noble *et al.,* 2011). Although the relationship between circulating concentrations of BDNF and body mass is unclear, reduced central expression is associated with obesity (Noble *et al.,* 2011). These studies show that appetite-related and inflammatory signals appear to mediate the impaired hippocampal neurogenesis resulting from chronic intake of refined sugars (at least in liquid form) at concentrations lower than those consumed by humans, and that these effects are independent of obesity.

#### 1.3.9.2 Saturated Fatty Acids (SFAs)

A study has also shown that feeding mice an HFD for two months, where 58% of energy was provided by SFAs, suppressed hypothalamic neurogenesis (see Figure 1.21; McNay *et al.,* 2012). This change was seen in the ARC and was linked to early apoptosis of newly proliferating cells. This decrease was partially reversed by calorie

restriction. The same research group determined that centrally infused CNTF stimulated proliferation within the hypothalamus of these obese mice and caused sustained weight loss. Many of the newly proliferating cells demonstrated functional phenotypes relevant to energy balance, such as leptin sensitivity, but the mechanisms that allow HFD to inhibit neurogenesis remained unclear (see Figure 1.22; Kokoeva *et al.,* 2005). However, the roles of circulating insulin and glucose have been linked with neurogenesis, as rodent models of diabetes show impaired hippocampal neurogenesis (Stranahan *et al*., 2008; Guo *et al.,* 2010).

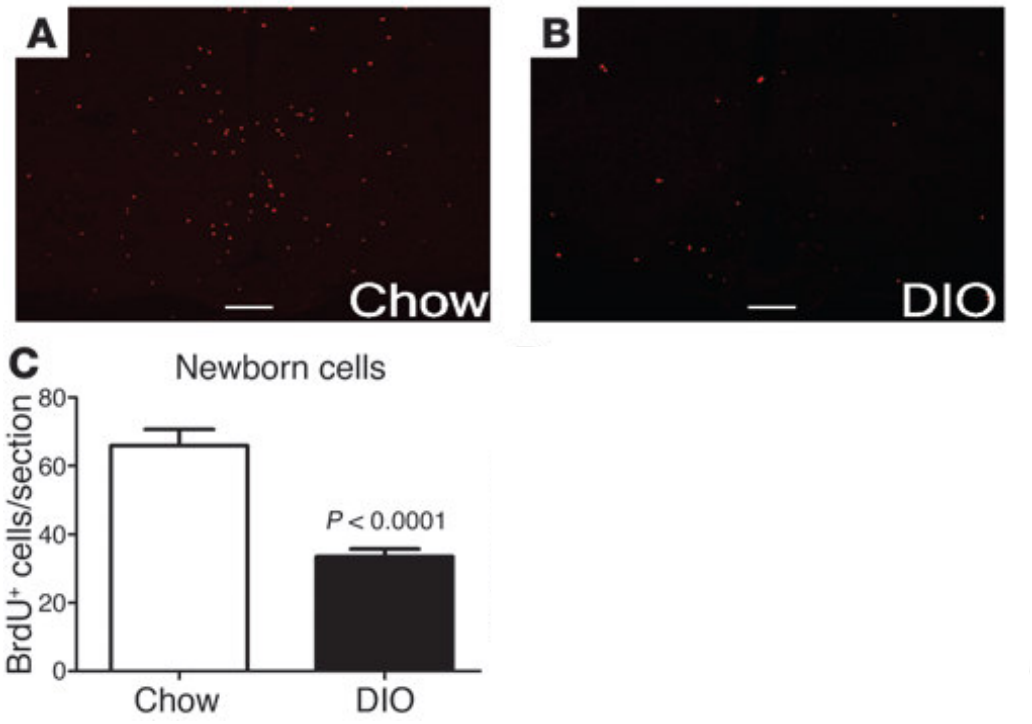



**Figure 1.21. DIO inhibits adult hypothalamic neurogenesis.** Sixteen-week-old mice were i.c.v. infused with BrdU for 7 days and brain tissue harvested 4 weeks later (A–C). The number of newborn (BrdU-labelled) cells was significantly reduced in the hypothalamus of DIO mice compared with that in lean controls. Scale bar: 100 μm. Data are mean ± SEM. n = 5 chow; n = 6 DIO. **Abbreviations**: BrdU= bromodeoxyuridine; DIO= diet-induced obesity. Source: McNay *et al.,* 2012. *J Clin Invest* **122**(1): 142-52.

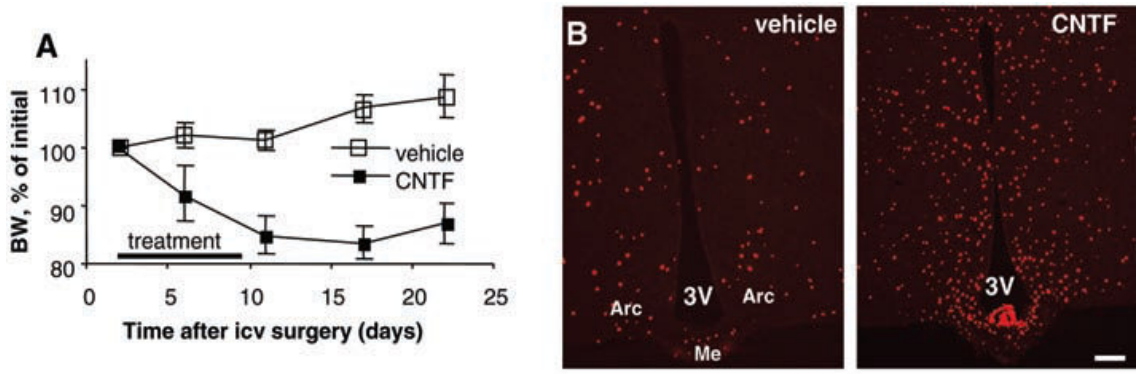



**Figure 1.22. CNTF reduces body weight long term and induces cell proliferation in the hypothalamus.** A: Mice were i.c.v infused for 7 days with BrdU (12 mg/day) in artificial cerebrospinal fluid alone or together with CNTF (0.75 mg/day). Body weight (BW) is shown as percentage difference from initial body weight (all data are mean ± SEM; *n*=5/group). B: BrdU-labelled cells in coronal sections of the hypothalamus at the level of the arcuate nucleus. **Abbreviations**: 3V= third ventricle; Arc= arcuate nucleus; BW= body weight; CNTF= ciliary neurotrophic factor; icv= intracerebroventricular; Me= median eminence. Source: Kokoeva *et al.,* 2005. *Science* **310**(5748): 679-83.

In contrast to these studies, Lee *et al.* (2012) demonstrated that young adult mice fed an HFD, providing a similar amount of energy from SFAs (60%), display active neurogenesis in the median eminence (see Figure 1.23a). Mice were fed up to 2.5 months of age (between postnatal days 5 and 75), after the diet had previously been supplied to their mothers. When this proliferation of cells was prevented, using irradiation (Figure 1.23c), weight gain was attenuated (Figure 1.23b) and energy expenditure and activity was increased (Figure 1.23d & e), suggesting a direct role for neurogenesis in body weight regulation. Interestingly, the rate of neurogenesis had increased significantly by postnatal day 75, indicating that HFD activation persists into adulthood, and suggests that it may modulate hypothalamic neural circuitry in later life.

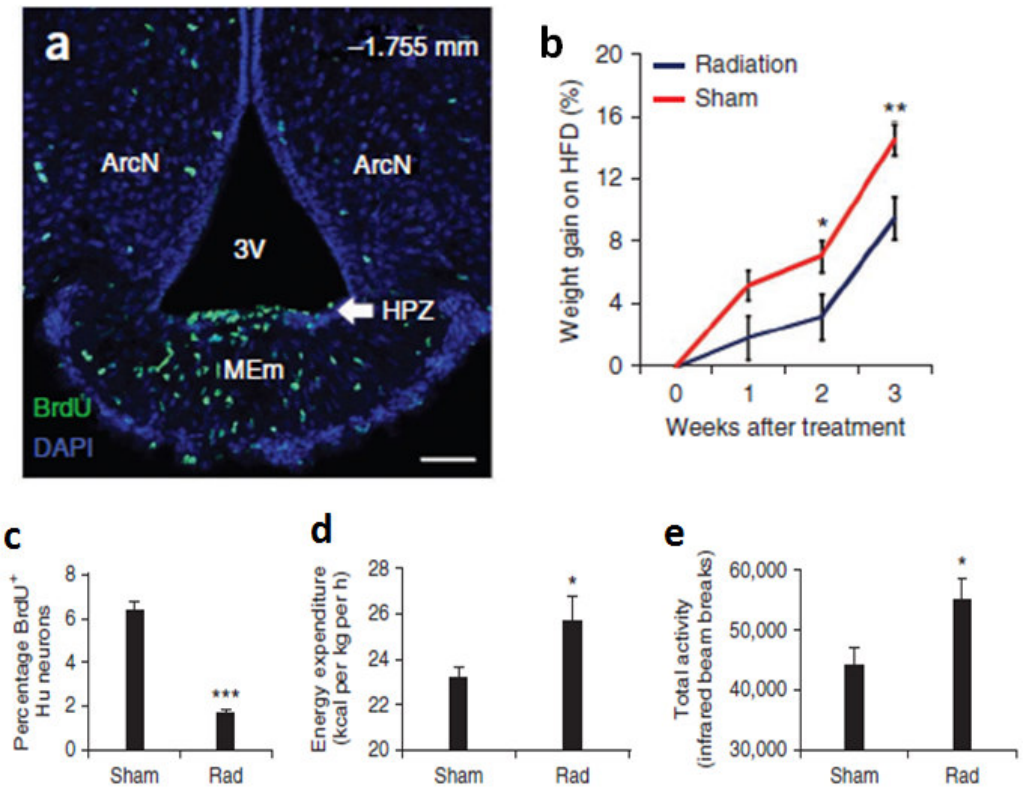



**Figure 1.23. Young adult mice fed an HFD display active neurogenesis in the median eminence.** P19 mice received BrdU (P10–P18), and hypothalamic sections were examined for BrdU immunostaining. (a) Hypothalamic proliferative zone (HPZ): enriched $BrdU^+$ cell population (green) along the median eminence (MEm) ependymal layer of 3V floor. Sections counterstained with DAPI (blue), a nuclear marker. Scale bar: 50 µm. (b) Attenuated weight gain in HFD-fed irradiated mice compared with sham controls ($n$ = 9). (c) MEm neurogenesis reduced in irradiated versus sham-irradiated mice ($n$ = 4). (d) Higher energy expenditure observed in irradiated mice ($n$ = 11). (e) Higher total activity observed in irradiated mice ($n$ = 11). Mean ±SEM; *P < 0.05, **P < 0.01; ***P < 0.01. **Abbreviations**: 3V= third ventricle; ArcN= arcuate nucleus; BrdU= bromodeoxyuridine; HFD= high-fat diet; HPZ= hypothalamic proliferative zone; MEm= median eminence; Rad= irradiated Source: adapted from Lee *et al.,* 2012. *Nat Neurosci* **15**(5): 700-2.

This idea is further supported by the work of Chang *et al.* (2008) who had observed that offspring of rat dams fed a high-fat diet (50% of energy from SFAs) displayed increased proliferation of neural stem cells (NSCs) and neural progenitor cells (NPCs) in the hypothalamus which prevailed at least until termination at postnatal day 70. In addition, enhanced differentiation and migration toward hypothalamic regions where these neurons ultimately expressed orexigenic peptides was also seen. This was linked with an increase in circulating lipids (triglycerides and free fatty acids) in the dams and offspring. Although a precise mechanism had not been determined, it was proposed that the purpose of this neurogenesis was to

prepare the juveniles for the increased food intake and high-fat diet preference they would probably show after weaning (Chang *et al.,* 2008).

In contrast, another study demonstrated that the number of adult hypothalamic NSCs in the VMH of three-month old mice was reduced and the process impaired following the chronic consumption of an HFD (58% energy from SFAs). Following an additional month of feeding this inhibition was even more severe. This was linked to the proinflammatory pathway involving IκB kinase β (IKKβ) and the downstream nuclear factor –κB (NF-κB) which mediates HFD-induced hypothalamic inflammation. Activation of the IKKβ and NF-κB pathways impairs the regulation of cell survival, growth, apoptosis and differentiation contributing to the neurodegenerative mechanism of obesity and related diabetes (Li *et al.,* 2012). This study differs from that of Lee *et al.* (2012), as short-term HFD feeding was reported to promote hypothalamic neurogenesis in juveniles, whereas long-term (4-month) HFD feeding described by Li *et al.* (2012) remarkably depleted hypothalamic NSCs and impaired neuronal differentiation. Li *et al.* suggest that whereas neurogenic upregulation by short-term HFD feeding may represent a compensatory reaction, long-term HFD feeding is detrimental for the cell fate of adult hypothalamic NSCs. They suggest that the neurodegenerative actions of chronic HFD feeding are attributed to IKKβ and NF-κB pathway overactivation, concluding that these NSCs are vulnerable to hypothalamic inflammation induced by chronic HFD feeding (Li *et al.,* 2012).

In contrast, another study has found no effect of HFD feeding on cell proliferation or neurogenesis in the hypothalamus or the hippocampus of rats fed a diet containing 60% of energy from SFAs for three months (Rivera *et al*., 2011). Despite the rats developing obesity, there were no differences in neurogenesis, when compared to a low-fat-fed control group, in the SGZ, SVZ or hypothalamus, consistent with similar concentrations of metabolism markers (plasma adiponectin, insulin and leptin concentrations) between the groups. The lack of effects may have been due to dietary composition, as sucrose and lard were present in both the high-fat diet and low fat control diets. This may have led to some metabolic dysfunction in the control group such that metabolic markers failed to differ.

### 1.3.9.3 Interaction with the Endocannabinoid System in Neurogenesis

Rivera's research was also attempting to find a link between cell proliferation and the endocannabinoid system in their model of diet-induced obesity. As mentioned previously, the endocannabinoids (intracellular signalling lipid-related molecules), play a regulatory role in controlling energy homeostasis through the activation of specific receptors in the hypothalamus and hippocampus which stimulate feeding (Piomelli, 2003; Pagotto *et al.,* 2006; Gamage *et al*., 2012). *In vitro* work and and the use of cannabinoid receptor knockout mice, has shown that endocannabinoids regulate neurogenesis in both the developing and adult nervous systems (Jin *et al*. 2004; Aguado *et al*., 2005; Galve-Roperh *et al.,* 2007; Oudin *et al.,* 2011). However, how the two phenomena were linked was unknown. Blocking the endocannabinoid receptors with antagonists such as rimonabant, the banned anorectic antiobesity drug reduces appetite, and therefore food intake, resulting in weight loss, increased energy expenditure and improvements in metabolic parameters (Matias & Di Marzo, 2004; Pavon *et al*., 2008; Scheen & Paquot, 2009). The rats from both dietary groups were treated with the cannabinoid receptor type 1 (CB-1) receptor agonist, AM251, which reduced food intake, caused weight loss and improved metabolism, as expected. However, accompanying reductions in circulating cholesterol, triglyceride and glucose levels were more pronounced in the HFD group and intriguingly, neurogenic change was stimulated only in this group. These changes were region-specific, with increases in neurogenesis observed in the SGZ, but reductions in the SVZ and contrasting with the findings of McNay *et al.* (2012), the hypothalamus. However, it is difficult to compare between studies utilising different species (mice *vs.* rats), diets and methods of inducing weight loss. The authors suggested that the endocannabinoid system is sensitised by HFD feeding, enabling the CB1 signalling system to adapt, in order to limit obesity-induced functional impairment in the brain. In addition, this adaptation is mediated by appetite-related signals. Overall, they concluded that cannabinoid modulation of cell proliferation in adult neurogenic regions is obesity-dependent (Rivera *et al.,* 2011).

In summary, it is difficult to make comparisons between all of these studies as they utilise different species, diets and methods for inducing weight loss; however, under most experimental paradigms using adult rodents, consumption of diets high in SFAs inhibits neurogenesis, a change that may be more nutrient- than

obesity-dependent. This alteration in neurogenesis within the hypothalamus and hippocampus may be mediated by changes in numerous metabolic signals. These may include triglycerides, glucose, and the trophic factors CNTF and BDNF. The full extent of at least some of their effects may depend on interaction with the endocannabinoid systems. Neuroinflammation, which, as described above, can also precede obesity, or develop independently of increased adiposity, during high-SFA consumption, is another phenomenon known to impair neurogenesis (Cota & Marsicano, 2011; Thaler *et al*., 2012). Whether it, in turn, is mediated by these same factors in this context remains to be tested. So far, chronic high-SFA consumption has been associated primarily with inhibition of neurogenesis, but the probable complexity of this relationship requires the careful design of studies comparing time course, fatty acid composition and concentration, model species, strain, age and neurogenic site.

#### 1.3.9.4 Polyunsaturated Fatty Acids (PUFAs)

In comparison to SFAs, PUFAs have been shown to improve VMH dysfunction in rodent models of metabolic syndrome (Pella *et al.,* 2011) through the normalising of appetite by restoration of neuropeptide concentrations (Das, 2008) and reducing hedonistic effects through the alteration of the dopaminergic and endocannabinoid systems (Golub *et al*., 2011). These alterations result in a reduction of appetite, enhancing satiety and reducing weight gain. Nonetheless, findings are generally inconsistent and in the human literature, only the addition of PUFAs as a supplement in the short-term has been examined (Lawton *et al*., 2000; Buckley & Howe, 2010). In general, the metabolic effects of long-term consumption of PUFAs incorporated into foodstuffs on the CNS needs to be clarified by utilising rodent models, specifically rat, under controlled conditions employing isoenergetic, purified and low-fat control diets (see Chapter 2).

As mentioned previously, the neuroprotection provided by omega-3 PUFAs is linked with membrane fluidity, neurotransmission, enzyme modulation and gene expression (Mazza *et al.,* 2007). The benefits of dietary consumption of omega-3 PUFAs have been demonstrated in both humans and rodent studies in the form of improved cognition in healthy individuals and those with neurodegenerative conditions such as Alzheimer's (Wu *et al*., 2004; Dyall, 2010; Su, 2010). It has been

proposed that these improvements occur through the stimulation of neurogenesis (Dauncey, 2009; Venna *et al.,* 2009; Matsuoka, 2011).

The omega-3 fatty acid, eicosapentaenoic acid (EPA), was the first to be associated with up-regulation of neurogenesis (Beltz *et al.,* 2007). This was shown in the lobster which has innately elevated levels of neurogenesis, making it easier to see neurogenic changes following an intervention. Increased cell proliferation was seen within the brain of lobsters consuming a diet enriched with PUFAs from brine shrimp or the microalgae *Spirulina,* which is often consumed by humans as a nutritional supplement (Beltz *et al.,* 2007). This increase manifested as an overall rise in baseline levels of neurogenesis, leading the author to conclude that the nervous system benefits from this nutritional addition. However, as no metabolic parameters were measured it is not known whether the improvement was directly stimulated by PUFAs or mediators of energy metabolism.

The patented LMN diet, rich in polyphenols and PUFAs, has been shown to enhance neurogenesis in the SGZ and SVZ in mice, but also to prevent cognitive decline seen with ageing and Alzheimer's disease (Valente *et al*., 2009; Fernández-Fernández *et al.,* 2012). Unfortunately, no metabolic factors were measured in either of these studies and therefore, it is impossible to make a connection between the PUFA consumption, metabolic factors and any potential influence they may have had on the enhancement of neurogenesis. Additional studies have explored the mechanisms behind the protective role of omega-3 FAs in learning and memory with ageing (Dyall *et al*., 2010). Supplementation with EPA and DHA reversed some of the decrease observed in hippocampal neurogenesis in elderly rats and prevented the reduction in hippocampal nuclear receptors (RARs/RXRs) involved in cell proliferation (Goncalves *et al*., 2009). Since the effects of the PUFA-enriched diet on energy metabolism were not investigated, no direct relationship was suggested for the mediation in neurogenesis. Instead the authors suggest that BDNF was the most likely candidate for controlling the observed changes, as its expression is known to be enhanced by omega-3 PUFA consumption and the nuclear receptor agonists, tamibarotene (Am80) and imipramine (Katsuki *et al.,* 2009; Venna *et al.,* 2009).

A study has established the link between adult neurogenesis and the consumption of the omega-3 PUFA, DHA, by showing that it stimulates

hippocampal neurogenesis in the dentate gyrus of adult rats (Kawakita *et al*., 2006). DHA is one of the main structural lipids in the mammalian brain, and plays a crucial role in the development and function of brain neurons (Barasi, 2007). The authors validated this finding through an *in vitro* study. By using primary culture of neural stem cells from 15-day-old rat embryos, and culturing these cells under different conditions with or without DHA, the study demonstrated that DHA is an essential molecule for cell differentiation by promoting cell cycle exit and preventing cell death (Kawakita *et al*., 2006). One study suggests that DHA enhances neuronal differentiation of NSCs, in part, by controlling the expression level of basic helix-loop-helix (bHLH) transcription factors and promoting cell cycle exit (Katakura *et al.,* 2009).

Furthermore, PUFAs have also been shown to activate the fat-sensing G-protein-coupled receptors (GPRs) present in tissues involved in the control of inflammation and energy metabolism (Oh & Lagakos, 2011; Talukdar *et al*., 2011). These include the receptors GPR40 and GPR120, which have been investigated for their role in PUFA-mediated reversal of SFA-induced hypothalamic inflammation associated with obesity (Cintra *et al*., 2012). The GPR40 receptor is involved in the upregulation of hippocampal neurogenesis required for improved memory function, and DHA has been shown to directly activate this receptor thereby suggesting another potential mechanism by which it may stimulate neurogenesis (Ma *et al.*, 2008; 2010; Yamashima, 2008).

In summary, evidence continues to surface that the chronic consumption of PUFA-enriched diets stimulates adult neurogenesis in brain regions associated with cognition and this may involve the GPRs. However, further work is required to determine fully the role of PUFA-induced changes in energy metabolism on neurogenesis itself, and within the hypothalamus, as the evidence for its role so far comes from non-dietary studies. There is evidence that omega-3 PUFAs exert their effects on cognition by affecting molecular events implicated in both synaptic plasticity and energy metabolism. BDNF is one proposed mediator between these two processes (Gomez-Pinilla, 2011), as PUFAs are known to enhance synthesis, secretion and intracellular signalling of this molecule, and therefore, may restore neurogenesis by this means (Wu *et al.,* 2004; Balanzá-Martínez *et al.,* 2011). As of yet, it has not been examined whether PUFAs exert their effects on body weight

homeostasis through changes in hypothalamic neurogenesis and whether these changes are mediated by BDNF. The slow turnover of hypothalamic neurons (Kokoeva *et al.,* 2007) alongside the technical limitations of labelling methods involving BrdU (Taupin *et al*., 2007; Cifuentes *et al*., 2011), as discussed further in Chapter 2, may explain why this relationship has not been explored further. If a relationship could be established, it would provide a neurobiological basis for the positive health benefits of dietary PUFAs, further supporting their role as a nutraceutical.

#### 1.3.9.5 Implications of Altered Neurogenesis

Determining how PUFAs regulate neurogenesis under conditions of altered energy metabolism, such as obesity, is key to fully appreciating their benefit as a nutraceutical. The potential for change in hippocampal neurogenesis has been suggested as a target for development of treatments for neurodegenerative disorders (Lindvall *et al.*, 2012), including those associated with changes in energy metabolism, such as diabetes (Balu & Lucki, 2009). The management of dietary intake is now viewed as an effective strategy to counteract neuronal disorders (Gomez-Pinilla & Gomez, 2011) and with further research, it may emerge that it also modulates hypothalamic neurogenesis to attenuate the development of obesity. This may encourage greater patient compliance than a pharmaceutical strategy. This is important to consider, as currently no dietary, pharmacological or lifestyle intervention has had any success in combating obesity (Lemieux & Lapointe 2008; Rössner *et al*., 2008). In summary, more research is required to fully quantify the relationships between individual macronutrients and neurogenesis. Once a more methodical approach to dietary intervention is adopted, and more information is acquired about the neurogenic capacity of the hypothalamus, particularly in the rat, scientists can start to consider possible mechanisms of action and translation to functional relevance in humans.

## 1.4 Summary

The literature review presented here has demonstrated the importance of regulating body weight for lifelong health and well-being as well as survival in general. A key factor involved in this regulation is the macronutrients we consume, some of which have been shown to have negative effects (SFAs and refined carbohydrates), and some, positive effects (PUFAs). We have seen that diets high in SFAs drive animals to overeat, overriding the effects of satiety hormones, and impairing neurogenesis, even before the onset of frank obesity. In contrast, separate lines of evidence show that dietary intake of PUFAs attenuates weight gain and stimulate neurogenesis. This raises the question of whether the control PUFAs exert over body weight is mediated by neurogenesis, as it appears to be with respect to SFAs.

### 1.4.1 Hypothesis

**Beneficial effects of PUFA intake on energy metabolism are mediated, at least in part, by stimulation of hypothalamic neurogenesis.**

The literature review above clearly shows how this complex hypothesis could not be tested fully in a three-year project. Indeed, an appropriate animal model, satisfactorily integrating valid phenotypes of PUFA feeding and hypothalamic neurogenesis would have to be established first. Most of the research conducted in this field has involved the use of mouse models, but as neurogenesis is known to be influenced by animal species and strain, there is a need to conduct further studies that investigate hypothalamic neurogenesis in rat. The technical aspects of this requirement are expanded upon in Chapter 2. Thus, even before an integrated model could be achieved successfully, two parallel aims would have to be satisfied:

### 1.4.2 Aims

1. to create a rational rat model of chronic PUFA consumption
2. to pilot methods for stimulating and observing neurogenesis in the hypothalamus of rat

# CHAPTER 2

## GENERAL METHODS

# General Methods

## 2.1 Animals and Treatment

### 2.1.1 Animal Experimentation

Laboratory animal experimentation in the United Kingdom is strictly governed by Home Office regulations, which have been devised to ensure that animals are treated humanely. Therefore, throughout the studies presented here, codes of conduct and treatments outlined in the Animal Scientific Procedures Act (1986) were adhered to. The Act sets out to provide clear definitions of a 'protected animal' and regulated scientific and experimental procedures which may be applied to said animal. It also serves to outline the conditions under which these protected animals are to be killed, and the humane methods by which this is to be carried out. It also states that before work can commence both a personal and project licence must be approved by the Home Office. Experimental procedures detailed under these licences have been tailored to the specific requirements of the studies described within this thesis.

One of the current aims of the Home Office is to encourage researchers to follow the 3Rs principle of replacement, reduction and refinement (Russel & Burch, 1959). This means that before a project licence is approved, applicants must have done the following, while still enabling their scientific hypotheses to be addressed:

(1) tried to find alternative means, not involving the use of animals (replacement);

(2) ensured through appropriate experimental design and use of statistics, that the minimum number of animals would be used (reduction); and

(3) justified their choice of animal, as being of the lowest sentience, which could still enable project aims to be addressed, and explaining how their suffering would be minimised while doing so (refinement).

The following sections explain how the studies presented in this thesis were designed and implemented with these principles in mind.

### 2.1.2 Use of Rodent Models in Studies of Body Weight and Feeding Regulation

Rodents respond to dietary interventions in ways that usefully model humans. These interventions range from caloric restriction to induction of obesity and in many cases, result in similar sequelae to those observed in humans (e.g. Buettner *et al.,* 2007; Panchal & Brown, 2011). In general, their small, manageable size enables studies, such as these, where reproducibility is desired, and replication is necessary to obtain statistical significance. With a relatively short lifespan, they allow for relatively rapid collection of data along multiple parameters of feeding and body weight metabolism. Advantages are that these can be examined at whole-body, integrated levels *in vivo*, and then at tissue and cellular levels *ex vivo*. *In vivo* measures can include energy intake in response to a variety of diet compositions and feeding schedules, resulting body weight, composition, adiposity and fat distribution, and behavioural indices, such as timing and volume of meals consumed. Concurrent intermittent body fluid sampling allows for monitoring of progression of dietary effects, through measurement of key endocrine and metabolic factors (e.g. West & York, 1998; Dobrian *et al.,* 2000; Keenan *et al.,* 2005; Buettner *et al*., 2007).

Rodents also allow for the convenient study of mammalian body-brain relationships and interactions, the complexity of which cannot be replicated in less sentient species or non-animal alternatives. This is particularly true when behavioural expression of these interactions is of interest, for example in the form of food selection and meal patterns (Farley *et al.,* 2003). Given that one of the main objectives of the work presented in this thesis was to examine associations between brain and body in the context of feeding, specifically, changes in hypothalamic cell proliferation in response to dietary fatty acids (Chapter 1, Section 1.4), animals which could model the sophisticated physiology of other mammals which have shown these relationships (Buettner *et al.,* 2007; Panchal & Brown, 2011), were essential to the work. Sub-mammalian species differ too fundamentally from humans in their systems physiology and brain structure to have more than limited relevance in this context. The alternative of human testing would have been unethical, as post-mortem tissue was required to examine changes in the brain at the level of resolution of individual cells.

These considerations also clearly show that *in vitro* work is not suitable in this research area because it cannot answer questions regarding phenotypic identification, number and regional distribution of cells in intact brain regions. It is self-evident that neither does it allow for development of a whole-animal dietary phenotype.

#### 2.1.2.1 Selection of Species

Rodents are the vertebrate group of lowest neurophysiological sensitivity which have also provided the best-characterised models of peripheral and central regulation of appetite (e.g. Schwartz *et al.,* 2000). Some mouse strains, such as the C57BL/6 can be more sensitive to dietary intervention, and weight gain, in particular, than commonly used outbred rats, such as the Wistar and Sprague-Dawley (Rossmeisl *et al.,* 2003). However, as models for examination of, and intervention in, already well-characterised feeding behaviours, they are less apt than rats (Anson *et al.,* 2003). Studies described here involved investigation of feeding behaviour expressed as meal patterns (see Section 2.4, below, and Chapters 3 & 5). Whereas there is no published evidence that mice have been used for this purpose previously, rats are commonly studied in such paradigms; for example, the behavioural satiety sequence (BSS; Halford & Blundell, 1996; Halford *et al.,* 1997; Cooper *et al.,* 2010) and studies of circadian rhythmicity of feeding (Kohsaka *et al.,* 2007; Hariri & Thibault, 2011). This provides an appropriate context into which results generated in this project can be placed.

#### 2.1.2.2 Selection of Strain

In investigating effects of dietary enrichment with PUFAs, it was important to incorporate the correct control diets into experimental design, as discussed in more detail below (Section 2.3.3). These included diets equally enriched in SFAs, and based on extensive literature, were predicted to induce obesity. Outbred rat strains, including Wistar and Sprague-Dawley, when fed such diets, become progressively more obese than their standard chow-fed littermates and develop insulin resistance and hyperleptinaemia (e.g. Storlein *et al.,* 1986; Chang *et al*., 1990; Kraegan *et al.,* 1991; Buettner *et al.,* 2006; Chen *et al.,* 2010; Hariri *et al.,* 2010), similar to obese humans (Kopelman, 2000; Woods *et al.,* 2003; Hariri & Thibault, 2010). They do not develop overt diabetes, and therefore, do not display β-cell failure or hyperglycaemia

(Levin *et al.,* 1997; Rossmeisl *et al.,* 2003; Collins *et al.,* 2004; Chatzigeorgiou *et al.,* 2009; Peyot *et al.,* 2010). This is typical of the prolonged period of impaired glucose tolerance observed in insulin-resistant obese humans, which can prevail for many years, preceding eventual development of frank diabetes (Ferrannini *et al.,* 2004). In this way, outbred rat strains with diet-induced obesity (DIO) are more appropriate models of human obesity and its associated metabolic dysfunction than monogenic strains, such as the Zucker or Zucker Fatty Diabetic (ZDF) rat. The current consensus is that most human forms of obesity are diet-induced and occur against a polygenic background; that is, they are multi-factorial, resulting from the interaction between several susceptibility genes and environmental influences, such as sedentary lifestyle (Speakman, 2004). Outbred rats in a given colony or sample population will show variable responses to diets of a given composition. No two randomly selected populations will gain weight to exactly the same extent or within exactly the same time period (Sclafani *et al*., 1976; Levin *et al.,* 1983; Levin & Sullivan, 1987; Chang *et al*., 1990; Levin *et al*., 1997; Reuter, 2007; Schariff & Ziv, 2009), as discussed in Chapter 1, Section 1.1.18.4. In contrast, the monogenic forms of obesity are due to single-gene defects, such as those resulting in the failure to produce a functional form of leptin or its receptor (Montague *et al.,* 1997).

In the studies detailed here, the Wistar rat was the outbred strain of choice because it had been shown in-house to reliably develop DIO when exposed to a highly-palatable diet (Pickavance *et al.,* 1999; 2000; Harrold *et al*., 2000a; Fatani *et al.,* 2007; Al-Qahtani *et al.,* 2008; Moore *et al.,* 2008). This would enable direct comparison of project data to previous in-house data for the purposes of monitoring body weight and metabolic change. Males were selected over females to avoid the potential influence of hormonal cycling on the parameters of interest and to enable comparison with the majority of the literature in the field (use of males being the convention). Rats were supplied by Charles River U.K. Ltd. (Margate, U.K.) at sexual maturity (about 6 weeks of age; 225-250 g), to restrict parameters to those of an adult model.

### 2.1.3 Experimental Design

Upon arrival, animals were stratified by body weight and randomly assigned to dietary (Part A; Chapters 3-5) or enrichment (Part B; Chapter 6) treatment groups, in factorial designs, in preparation for studies with the following generic schedule (Fig. 2.1).

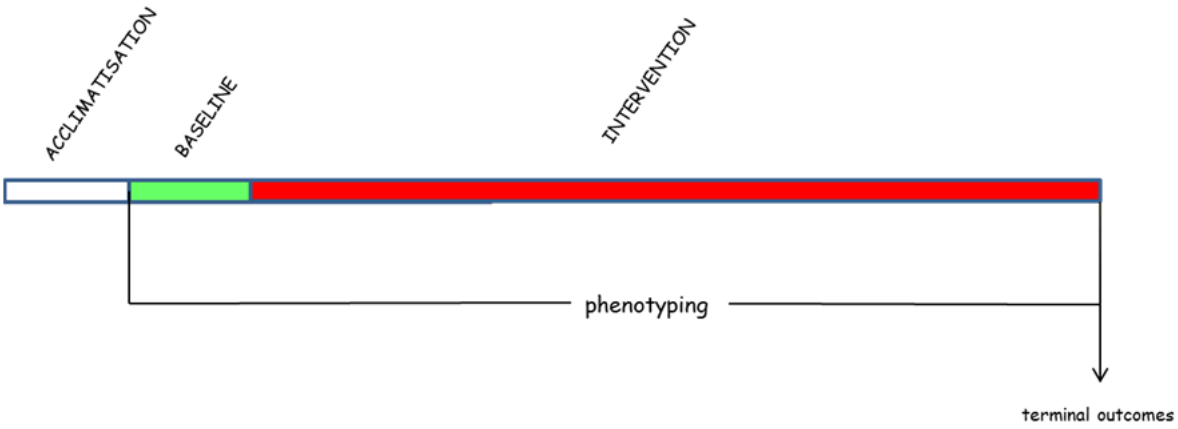



**Figure 2.1. Study timeline.** After assignment to treatment groups, rats acclimatised to the new environment of the Biomedical Services Unit, University of Liverpool, for 1-2 weeks. Baseline measures, including body weight and 24-hour food intake were then collected, along with an initial blood sample, prior to any intervention. Interventions included exposure to diets of differing fat content (Chapters 3-5 & 7) or simple environmental enrichment in the form of a play tube (Chapter 6). Phenotyping involved continued periodic collection of morphometric, biochemical and behavioural (meal pattern) data (Chapters 3 & 5) until termination. Terminal outcomes were measures capable of being taken just prior to death by perfuse-fixation (including final body weight, food intake, blood samples, and later measures from harvested tissues). A more detailed summary of workflow and outcome measures can be seen in Figure 2.2.

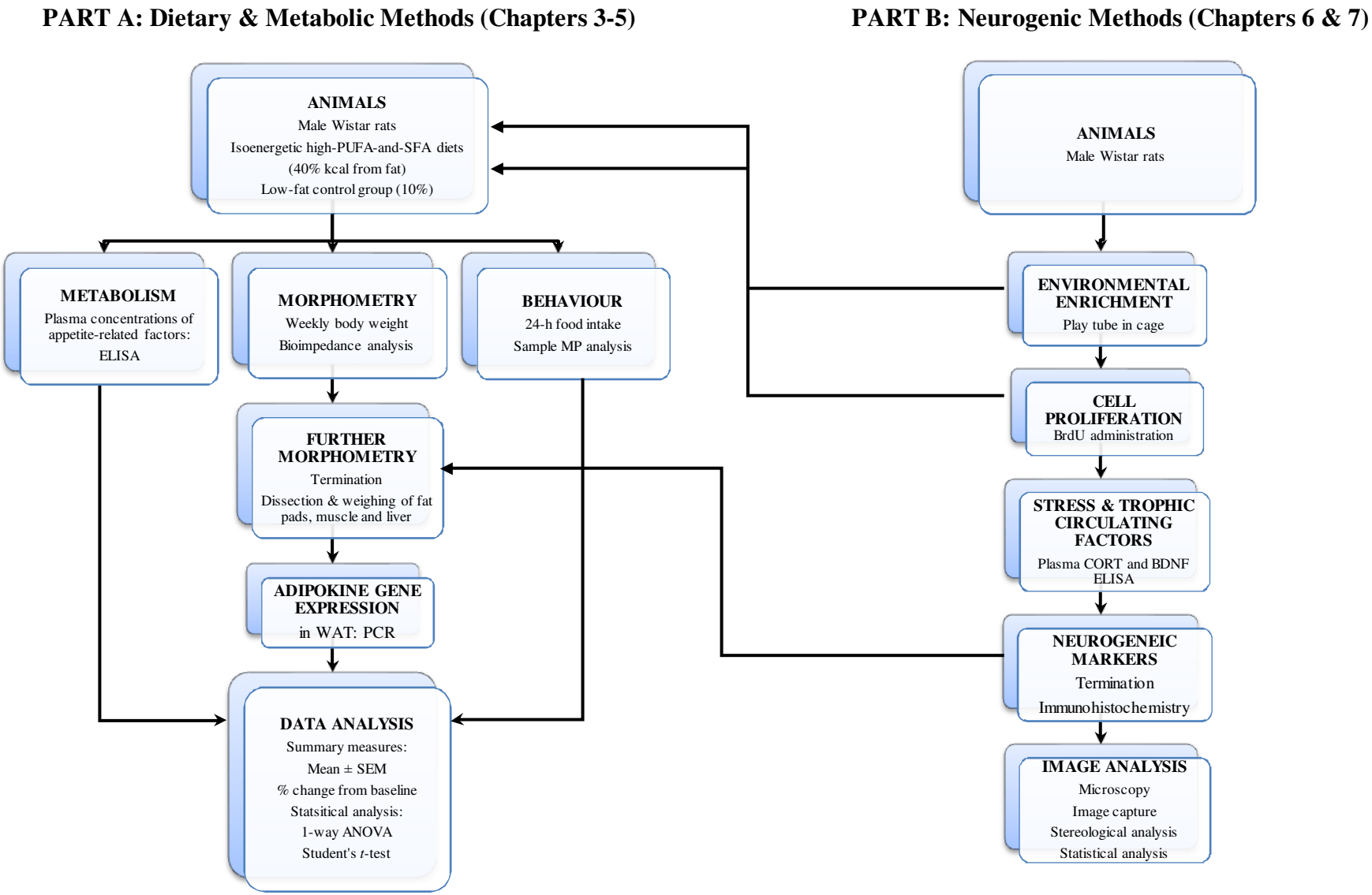



**Figure.2.2. Workflow and outcome measures**. Some or all of the outcome data shown here were collected at the end of each of the studies outlined in Chapters 3-7 depending on its purpose. Work in Chapters 3 and 6 were concurrent (see Figure 2.21). **Abbreviations:** ANOVA=analysis of variance; BDNF= brain-derived neurotrophic factor; BrdU= 5-bromo-2-deoxyuridine; CORT= corticosterone; ELISA= enzyme-linked immunosorbent assay; MP = meal pattern; PCR= polymerase chain reaction; PUFA= polyunsaturated fatty acid; SEM= standard error of mean; SFA= saturated fatty acid; WAT= white adipose tissue.

### 2.1.4 Sample Size Determination

#### 2.1.4.1 Power Analysis

Power analysis, which is defined as the probability of finding a real biological difference if it exists, is often used to determine the number of animals required in a treatment group. However, there are often occasions when it becomes difficult to use a power analysis because there is no information on inherent variability, such as standard deviation, and because it is difficult to specify the expected size of effect from variables (Grafen & Hails, 2002). An alternative method for appropriate sample size determination was proposed by Mead (1988). The method is considered appropriate for comparative experiments which can be evaluated using analysis of variance (Grafen and Hails, 2002; Kutner *et al.,* 2004), such as exploratory experiments and complex biological experiments with several factors and treatments (such as those described in this thesis). Inherent study complexity (involving several dependent variables, such as diet-type) warranted the use of Mead's resource equation over the more commonly used power analysis to determine how many animals should be assigned to each group (*n*).

#### 2.1.4.2 Mead's Resource Equation

Dietary effects can be influenced by many factors (e.g. strain, age), and masked by many sources of variation (e.g. palatability and texture). In the design of animal experiments, Mead's resource equation is used to first design pilot studies which identify these sources, and then to determine the correct numbers of animals needed to give sufficient statistical power for full-scale studies. This then gives confidence in the findings, such that repetition of studies, and hence, use of additional animals is unnecessary (Festing *et al*., 2004).

Mead's resource equation is as follows:

**E = N - T – B**

In an example taken from this project,

E = error degrees of freedom (df) *(between 10 and 20)*

N = total df = total number of experimental units *(animals)* - 1

T = treatments df = number of treatment *(diet)* combinations – 1

B = blocks df = number of blocks *(cage runs*)* – 1

**The parameter of meal pattern was measured by automated cages (Section 2.4, below). Availability of only 4 cages required rats to be monitored in groups of 4, with group members counterbalanced across each cage position and set of automated sensors. A "monitoring session", therefore, constituted a source of variation that had to be blocked.*

Selecting 8 animals/group,

E = (8 rats/group x 2 treatment groups -1) – (2-1) – (4-1) = 15 – 1 – 3 = 11

Mead's resource equation states that E should fall between10 and 20; i.e.$10 < E < 20$. Therefore, for this example, $10 < 11 < 20$. When selecting 7 animals/group,

E = (7 rats/group x 2 treatment groups -1) – (2-1) – (4-1) = 13 – 1 – 3 = 9,

where $9 < 10$.

Therefore, an attempt to address the 3Rs by reducing animal numbers to seven per group would fail to satisfy E, but eight animals per group would be an optimal minimum sample size to reveal a biological effect, if one exists.

### 2.1.5 Assignment to Treatment Groups & Counterbalancing

Rats were assigned to treatment groups so as to address issues of logistics and potential bias, thereby minimising sources of variation. Stratification involved grouping the animals into categories predefined by body weights ranging from lowest to highest. They were then assigned to treatment groups to ensure that group mean starting weights were not significantly different.

Counterbalancing position of rats' cages across cage racks, according to their assigned treatment group, controlled for variation arising from exposure to the scent of a neighbour's diet or degree of exposure to overhead room lights, for example. The cage array also corresponded to the order in which animals were to be terminated (Figure 2.3). This controlled for ultradian variation in concentrations of circulating factors in terminal blood samples.

| Batch A (TD1) | | | | | | Batch B (TD2) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **ID** | **Diet** | **ID** | **Diet** | **ID** | **Diet** | **ID** | **Diet** | **ID** | **Diet** | **ID** | **Diet** |
| **114** | **PUFA** | **117** | **Control** | **120** | **SFA** | **123** | **PUFA** | **126** | **Control** | **129** | **SFA** |
| **115** | **SFA** | **118** | **PUFA** | **121** | **Control** | **124** | **SFA** | **127** | **PUFA** | **130** | **Control** |
| **116** | **Control** | **119** | **SFA** | **122** | **PUFA** | **125** | **Control** | **128** | **SFA** | **131** | **PUFA** |

**Figure 2.3. Example of a cage array.** This illustrates the configuration employed in studies of dietary intervention (Chapters 3-5), but the same principles were applied in studies of environmental enrichment intervention (Chapter 6). For logistical reasons, animals were (1) split into two equivalent batches for which sampling and termination were separated by *x* days, and (2) Terminated across two days. Diets were enriched in PUFAs or SFAs. Control animals received a standard, low-fat diet. **Abbreviations:** ID = rat identification number; PUFA = polyunsaturated fatty acid; SFA = saturated fatty acid; TD = termination day.

A counterbalanced design was also applied to the use of the automated cages used for meal pattern data acquisition. The availability of only four cages limited the number of rats which could be monitored at any one time, such that several 'runs', in a configuration similar to that shown below (Fig. 2.4), would be required to analyse all animals.

**Batch A**

**Day 1**

| **Cage 1** | **Cage 2** | **Cage 3** | **Cage 4** |
|---|---|---|---|
| Diet 1 | Diet 2 | Diet 3 | Diet 1 |

**Day 2**

| | | | |
|---|---|---|---|
| Diet 2 | Diet 3 | Diet 1 | Diet 2 |

**Day 3**

| | | | |
|---|---|---|---|
| Diet 3 | Diet 1 | Diet 2 | Diet 3 |

**Batch B**

**Day 1**

| | | | |
|---|---|---|---|
| Diet 3 | Diet 1 | Diet 2 | Diet 3 |

**Day 2**

| | | | |
|---|---|---|---|
| Diet 1 | Diet 2 | Diet 3 | Diet 1 |

**Day 3**

| | | | |
|---|---|---|---|
| Diet 2 | Diet 3 | Diet 1 | Diet 2 |

**Figure 2.4. Example of counterbalanced configuration used for meal pattern data acquisition.** Diet group members were counterbalanced across each cage position and set of automated sensors. In effect, meal patterns were 'sampled' at predetermined intervals, just as other data sets collected over the time course. Rats were removed from home cages and placed in automated cages for 24- to 72-h periods. In a 72-h run, days 1-3 were consecutive.

### 2.1.6 Housing and Maintenance

Rats were singly housed, which allowed for individual food and water intake measures. The ability to collect individual data is essential for accurate measurement of feeding parameters, but can impoverish the cage environment. Therefore, the risk of social isolation, was controlled by the use of high-topped cage lids, enabling visual and olfactory contact with neighbours, and the physical environmental was enriched with a PVC 'play' tube in the case of all rats dietary studies (Chapters 3-5 & 7) and experimental rats in enrichment studies (Chapter 6).

Rats were housed on shavings in solid-bottomed cages cleaned out once weekly at the same time. They were maintained at 22 ± 2°C on a 12-hour light-dark cycle (lights on at 08.00) and had free access to tap water throughout each study. They had *ad libitum* access to standard laboratory chow (3.4 kcal/g; CRM, Special Diet Services, Horley, UK) for a seven-day minimum acclimatisation period prior to the start of studies. In enrichment studies (Chapter 6), this continued throughout. In dietary studies (Chapters 3-5, 7), rats then received specialised formulated diets (Research Diets Inc™, NJ, USA; see Section 2.3). Post-acclimatisation, food and water intake were measured daily and body weight weekly to monitor metabolic change in response to experimental diets (Chapters 3-5, 7) or as a general health check in response to intake of cell proliferation tracer (Chapter 6). Although recorded, water intake data did not show any differences between treatment groups and are not presented here. Rats were weighed using an animal weighing density determination check system which compensates for their movements (Precisa, Precision BJ2100D, Milton Keynes, Bucks., U.K.).

# PART A: Dietary and Metabolic Methods

## 2.2 Blood Sampling for Measurement of Circulating Hormones and Lipids

Before blood sampling, rats underwent an overnight fast to reduce variability in plasma concentrations of hormones and metabolites. This took the form of providing about 50% of the rats' average overnight intake at the end of the day.

Blood was obtained for diagnostic assay by extraction from the tail. This was performed on a weekly or fortnightly basis during periods of diet exposure (Chapters 3-5), or on termination (Chapter 6). Rats were first lightly anaesthetised with gaseous isoflurane (2% for 5 minutes) and the extreme tip of the rat's tail was removed, avoiding the terminal vertebrae. Known as tail-tip amputation, this allowed for repeated sampling from the same rats in studies following the progression of metabolic changes. It enabled quantities of blood (approx. 250 µl), yielding plasma in sufficient volume for assay, to be quickly and painlessly removed from the rat. A scab would form at the tail-tip overnight and was simply snipped off for the next sampling a few weeks later and blood collected directly. At the time of collection, whole-blood glucose concentrations were measured using a hand-held blood glucose monitoring system and glucose-oxidase strips (Ascensia Breeze 2, Bayer HealthCare, Leverkusen, Germany).

Blood was collected in lithium-heparin microfuge tubes and centrifuged at 6000 revolutions per minute (rpm) for ten minutes (min) (Sorvall Legend Micro 17, Thermo Scientific, Waltham, USA). Plasma was then separated and stored at -20°C for later measurement of hormones and lipids by enzyme-linked immunosorbent assay (ELISA) or diagnostic assay (see below for descriptions of these assays: See Section 2.6).

### 2.2.1 Anaesthesia

The small body size of the rat makes intravenous (i.v.) injection difficult, and drugs are usually administered by intraperitoneal (i.p.) routes. If these routes are used it is not possible to administer a drug gradually to effect, and the anaesthetic must be given as a single calculated dose. Because of the wide variation in drug response between different strains of rat, between male and female animals, and between

individuals, it is best to use a drug, or drug combination, providing a wide margin of safety.

The anaesthetic combination of choice for rats detailed here is medetomidine (Domitor™) and ketamine (0.6 ml/kg and 0.4 ml/kg, respectively). Medetomidine is a potent non-narcotic $\alpha_2$-adrenoreceptor agonist which produces sedation and analgesia. Ketamine is classified as an NMDA receptor antagonist, and has opioid-like effects. Medetomidine is often used in combination with opioids, in this instance ketamine, to induce complete relaxation in the rat, and is given intraperitoneally (i.p.) to produce general anaesthesia. The combination provides a good degree of muscle relaxation, with the effects lasting approximately 20-40 minutes (Flecknell, 1996). This combination was used for the purposes of perfuse-fixation in all studies described here. Administration of pre-anaesthesia by inhalation of isoflurane was also used to reduce the effects of stress, which could have affected circulating concentrations of corticosterone, one of the outcomes of interest, and levels of neurogenesis (Chapter 6, Section 6.3).

The number of anaesthetic exposures was minimised by performing bioimpedance (described in Section 2.5.2) and blood sampling, both short procedures, under the same anaesthesia.

## 2.3 Dietary Studies

As discussed in Chapter 1, dietary studies, both human and rodent model, are largely in need of greater systematisation and application of appropriate controls. The following sections describe how this was addressed in this project.

### 2.3.1 Choice of Commercial Diets

It was determined that the primary requirement of the chosen supplier would be the ability to modify fatty acid (FA) content of the rodent diets in such a way that (1) the specific fatty acids desired could be incorporated into a pelleted chow (so as to be compatible with wire mesh feeding hoppers); (2) any two high-fat diets (HFDs) would be equivalent in energy content (isoenergetic); and that, (3) along with any standard, low-fat control diet (LFD), would have, as far as possible, identical nutrient compositions, apart from the constituent (fatty acid) of interest. It was discovered that Research Diets, Inc.™, (NJ, USA; hereafter, 'Research Diets'; Figure 2.5), as opposed to other potential suppliers, such as Purina® and Teklad™, were able to meet these criteria. Their diets are referred to as OpenSource® (hereafter, 'OpenSource'), which simply means that they have 'open' formulas (i.e. that are published and available to the scientific community) and that each nutrient is supplied by a separate, purified ingredient.


**Figure 2.5. Examples of solid pellet diet products manufactured by Research Diets.** Diets are provided as nutritionally complete to prevent nutritional imbalance. Different dietary compositions incorporate different food dyes for identification purposes. Fatty acid content above 60% requires that the diet be provided in paste form. Source: www.researchdiets.com.

Because the purified ingredients used in these diets are refined materials, as opposed to the less refined ingredients found in standard chow provided by other suppliers, the company can define the nutritional requirements of animals by selectively removing one nutrient at a time from the diet. This allows for endless modifications and researcher specifications. Previously, high-fat diets have been made by adding high levels of fat to grain-based chow diets. Critically, this dilutes other essential nutrients such as vitamins and minerals (Cybulsky *et al.,* 1999). Many diet manufacturers claim to offer the same "open source" formulas as Research Diets. However, although their formulas are open, as defined above, they often vary in their sources of ingredients, or vitamin and mineral mixes. Currently, the only way to ensure the consistency of diets across studies is to purchase from Research Diets (Gajda *et al.,* 2007).

### 2.3.2 Modification of Diet Composition

The main issues associated with dietary studies are the correct selection of macronutrients (type and concentration) and their appropriate controls. Numerous modifications can be made to OpenSource diets to satisfy these requirements, ranging from alterations in macronutrient energy source (e.g. carbohydrate *vs.* fatty acids) to removal of micronutrients (vitamins and minerals) in order to model dietary deficiencies and define requirements (Gajda *et al.,* 2007). The studies in this project involved not only modification of fatty acid content, but also fatty acid type, in a base, or standard control, diet; for example, replacing a baseline concentration of polyunsaturated fatty acids (PUFAs) sourced from soybean oil, with one of saturated fatty acids (SFAs) sourced from coconut oil, sufficient to quadruple the energy contributed by fat (Chapter 5). One of the parameters commonly adjusted by Research Diets during formulation is the elevation of sucrose concentration to allow researchers to study the development of insulin resistance, the implications of which are discussed in Chapter 3 (Section 3.4.4). All dietary modifications described here were made to the original Research Diets 'low-fat control' D12450B (Table 2.1).

**Table 2.1. Nutritional breakdown of low-fat control diet (D12450B; Research Diets, Inc.)**

| **Product** | **D12450B** | |
|---|---|---|
| | gm% | kcal% |
| Protein | 19.2 | 20.0 |
| Carbohydrate | 67.3 | 70.0 |
| Fat | 4.3 | 10.0 |
| Total | | 100.0 |
| Kcal/gm | 3.85 | |
| | | |
| **Ingredient** | | |
| Casein, 80 mesh | 200 | 800 |
| L-Cystine | 3 | 12 |
| Corn Starch | 315 | 1260 |
| Malodextrin 10 | 35 | 140 |
| Sucrose | 350 | 1400 |
| Cellulose, BW200 | 50 | 0 |
| Soybean oil | 25 | 225 |
| Lard | 20 | 180 |
| Mineral Mix S10026 | 10 | 0 |
| DiCalcium Phosphate | 13 | 0 |
| Calcium Carbonate | 5.5 | 0 |
| Potassium Citrate, 1 $H_2O$ | 16.5 | 0 |
| Vitamin Mix | 10 | 40 |
| Choline Bitartrate | 2 | 0 |
| FD & C Yellow Die No.5 | 0.05 | 0 |
| **TOTAL** | 1055.05 | 4057 |

Complete nutrient breakdown is provided in gm% and kcal% format. Macronutrients with the potential for modification are highlighted: Protein = green; Carbohydrate = blue; Fat = orange/yellow. For the studies described here, it is the fat source which was altered. Source: www.researchdiets.com.

### 2.3.3 Control Diets

Rodent diets typically fall into two categories, the standard chows (maintenance diets) and the purified OpenSource diets. The endless capacity for dietary modification clearly supports the concept that OpenSource diets are superior, not only as experimental diets, but also as their controls. Standard chow diets are commonly used as the control diet as they are inexpensive, yet provide suitable nutrition. However, it is important to consider that these chow diets contain plant material with varying nutritional content (Thigpen *et al.,* 1999), therefore, making it

impossible for each batch to be the same (Wang *et al.,* 2005). This makes their use in dietary studies, where nutritional intake requires strict control, difficult. Additionally, most chow formulas are closed to the public and modifications are unreliable, as each plant ingredient contains numerous nutrients (Brown *et al.,* 2001).

Research Diets formulate the experimental and matching control diets to meet the specific study needs, including the animal model, desired phenotype and dietary requirements. It is important that when modifications are made, the remainder of the diet remains identical to the unmodified control. This allows for comparisons across the experimental groups, as only one diet component changes. This principle is simple to understand when it comes to removing or adding components that do not have caloric content, such as vitamins and minerals. However, when macronutrient (protein, carbohydrate, or fat) content is modified, it becomes more difficult, and it is, therefore, necessary to look at the ratio of a nutrient per calorie of diet, as animals tend to eat for calories, not for weight. This is seen when rodents that are used to consuming a diet low in fat begin eating less food by weight (grams) when they are switched to a high-fat diet (HFD). This further explains why diets of varying caloric (energy) density should be formulated to have similar nutrient-to-calorie ratios, ensuring that, per calorie of intake, animals consuming diets of varying caloric density will still receive the same amount of nutrients (Ricci & Ulman, 2008).

### 2.3.4 Isoenergetic Diets

There are two types of control diets, one which controls for normal growth and the other which controls for energy content (isoenergetic). Isoenergetic diets which are identical in all but the FA type are nutritionally complete and take the form of solid pellets, thereby controlling for variability in total energy intake and texture. Commonly, when comparing a modified diet high in a particular FA (e.g. a PUFA), results are compared to another HFD of identical energy content but different FA type (e.g. a SFA). These diets then act as controls for one another. A third group, or second control, is the included, wherein animals are fed standard (low-fat) chow. This groups acts as a control for obesity induction, demonstrating normal weight gain and food intake. A common mistake made by many researchers is the use of a standard chow diets as a low-fat control in dietary studies. As mentioned above, standard laboratory chows contain plant material with varying nutritional content and

therefore, diet formulations will vary from batch to batch, introducing additional factors, which may confound results (Ricci & Ulman, 2008). Ideally, a control diet should match the experimental diet not only in energy content (i.e. be isoenergetic), but all other ingredients as well, except for the modified compound of interest.

### 2.3.5 Varying Fatty Acid Composition

When formulating Research Diets the LFD control, the protein requirement is commonly met by the milk protein, casein, carbohydrates are supplied by corn starch and sucrose, soybean oil provides the fat, and cellulose supplies the fibre (Table 2.1). Formulation of the diets required for this project involved modification of some of these standard ingredients to meet experimental aims, as described in Chapters 3-5. Thus, varying fatty acid composition would enable exposure of animals to differing fatty acid types and elevated concentrations. However, in order to keep overall energy content constant, concentrations of other macronutrient constituents also had to be altered. Importantly, vitamin and mineral mixes specific to rodents were always added at concentrations to ensure nutritional adequacy.

Thus, in project studies (Chapters 3-5, 7), isoenergetic HFDs, equally enriched in SFAs or PUFAs, were compared for effects on a range of phenotypic and metabolic parameters, including novel aspects of feeding behaviour (meal patterns). Diets were formulated to provide 40% of energy from fat. Consultation of the literature indicated that this was the highest concentration which could be produced in pellet form, and which also would be likely to stimulate both metabolic and neurogenic change. Pelleting was important for preventing loss of diet through the hopper, a disadvantage of soft, very high-fat diets (e.g. providing 60% of energy from fat), which can crumble (Fatani *et al.,* 2007). This ensured energy intake measures remained accurate. The outcomes of HFD consumption were compared to those of a low-fat control diet (providing 10% of energy from fat) with an otherwise equivalent nutrient composition. All were variations of the base diet composition shown in Table 2.1. Development of the individual diets used in this project is outlined in Chapter 3 and 5. Full nutritional breakdowns and respective fatty acid profiles can be found in the Appendix I-IV.

## 2.4 Meal Pattern Analysis

### 2.4.1 Why Measure Meal Patterns?

The measurement of periodic food intake over a set unit of time, such as 24- hour intervals, is defined by the researcher and not the experimental animal. From this type of data collection, the amount of food consumed during the time period is calculated by difference from a previous measurement. Most commonly, a known amount of diet is given to the animal, and after a defined time elapses (set number of hours, days), the food is re-weighed and the amount consumed is calculated. Feeding episodes, bouts, and meals cannot be distinguished by this periodic manual collection of data. These types of methods are time consuming and cannot be carried out continually. People collecting the periodic data can also affect the animals' behaviour. The act of moving the animal and its food to weigh it can interrupt a meal, affecting food intake. Therefore, the more manual measures taken in a day, the less likely the data is to be a true representation of the animal's natural feeding behaviour. In comparison, episodic food intake is the automatic recording of the animal's individual eating episodes. Each eating episode is recorded as a unique record including the start time, duration, and amount consumed. From this information, meal records can be constructed. Specialised cage systems have been developed to automate this process, saving labour and limiting disruptive human contact with feeding animals, as well as allowing undisturbed and continuous food and water weighing across nocturnal and diurnal time periods (Ulman *et al.,* 2008). Much can, and has, been learnt about total periodic food and water intake using systems based on this principle. This includes information on the nocturnal and diurnal control of appetite, as well as characterisation of circadian rhythmicity of feeding (Hairiri & Thibault, 2011).

### 2.4.2 Feeding and Meal Pattern Parameters

Obesity has many correlates, including disrupted (abnormal) meal patterns (MPs), which have been modelled in rodents. These not only include changes in intake (volume), but also meal duration, frequency, distribution across the day and night (temporal sequence), and inter-meal intervals (Farley *et al.,* 2003; Farshchi *et al.,* 2005). Thus, obesity is a disorder expressed on several levels, ranging from genetic to behavioural (Vandenbroeck *et al*., 2007). The advantage of studying MPs in this

kind of detail gives us a greater understanding of how appetite is regulated by signals from the brain and body tissues, which switch on or off at different times in relation to when meals are taken (Leidy & Campbell, 2010). In addition, the nutrient composition of food, and its overall energy density, control meal size and feelings of fullness (satiety), determine when the next meal will be consumed. Automated cage systems aid in generating extensive data for each animal on simultaneous progression of several subtle and otherwise difficult-to-measure, aspects of feeding and drinking, such as meal volume and periodicity (Ulman *et al.,* 2008). It also allows for the monitoring of day-to-day rodent health when administering substances.

The most significant advantage of these types of studies is both practical and ethical: Each individual animal generates a large amount of data, enabling smaller, more manageable and quickly processed experiments, and hence a higher turnover of research outputs; reducing the number of animals to answer research questions also importantly works toward fulfilling the 3Rs.

Acquiring data on MPs with automated cages helped to fully characterise the dietary model described within this project. The measurement of MPs was an integral part of these studies as it provided important behavioural information on the effects of eating a diet high in fat, especially as MP signatures for diets high in PUFA content had yet to be determined in a rat model.

### 2.4.3 The LabMaster™ System

Crude measures of feeding and drinking behaviour were taken by manual weighing of food hoppers and water bottles at the same time of day every 24 hours throughout the duration of study. Detailed analysis of feeding (meal) and drinking patterns was carried out periodically by transferring rats to automated cages largely equivalent to the home cages used for daily intake measures. These were connected to data acquisition software (LabMaster™, TSE Systems, Hamburg, Germany; hereafter referred to as 'LabMaster'), allowing for the collection of a series of weights as raw data measurements, which were then analysed using various parameters to calculate aspects of feeding, including day/night volume, periodicity and satiety. (As water intake did not alter in response to any dietary interventions, data are not presented in this thesis.)

The LabMaster system (see Figure 2.6) was chosen for MP analysis because it is specially designed to monitor metabolism and behaviour of small laboratory rodents. This particular system was chosen over other possible alternatives, such as BioDAQ® (Research Diets Inc.™, NJ, USA) and the Oxymax Lab Animal Monitoring System (Columbus Instruments, OH, USA) because it is the only cage and software system which performs its own initial basic meal pattern calculations for parameters such as feeding rate. Furthermore, LabMaster is modular and can be expanded in future to include more cages and attachments for measuring additional locomotor activity parameters and ultimately, full metabolism (e.g. body weight, calorimetry, activity monitoring etc.).

**Figure 2.6. LabMaster™ cage system.** Each cage is equipped with a suspended food hopper and water bottle attached to sensors from which changes in weight are transduced to raw data collected across time and which can then be analysed according to various feeding parameters. Source: www.tse-systems.com.

Solid-bottomed cages were used in these studies to provide an environment for monitoring unbiased, stress-free animal behaviour. Drinking and feeding behaviour was measured by high-precision weighing sensors fixed to the tops of the cage lids (Fig. 2.6) and connected by cables to a computer which transduced mechanical weight change information into raw numerical data. This was then exported in text and CSV file formats to Microsoft Excel files, with which both are compatible, and analysed by statistical programs. The LabMaster system itself was

capable of carrying out limited meal pattern analysis, which aided monitoring of experimental progress while studies were underway (Fig. 2.7).

Rats were monitored in these cages intermittently for 24-72 hours, depending on the study in question, and were given *ad libitum* access to diet and water.

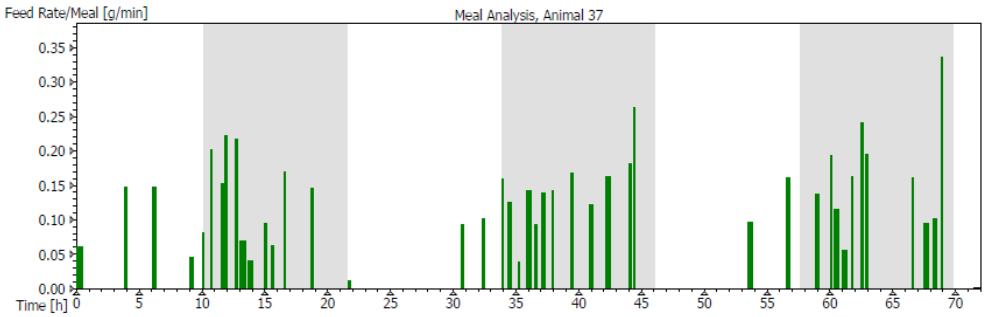



**Figure. 2.7. Example of graphical data presentation by LabMaster™ software.** The graph displays information on feeding rate, showing individual meals (green bars) over a three-day period (*x*-axis units in hours). The *y*-axis shows feeding rate in grams consumed per minute. Nocturnal phases are highlighted in grey.

### 2.4.4 Defining Meal Patterns

#### 2.4.4.1 Defining a Meal

The LabMaster system allowed uninterrupted, undisturbed recording of individual meals for each animal. Commonly, a meal is defined as a single episode of uninterrupted feeding, with a specific start time, feeding duration, and quantity consumed. During the studies described here, the weight of the food hopper was recorded at 10-minute intervals, as recommended by TSE Systems as a feasible resolution for the collection of feeding data; i.e. providing sufficient detail while avoiding generating unmanageable quantities of data. A meal was defined as a change in hopper weight of ≥0.1 g, separated from other meals by >10 minutes. No spillage was noted during these studies. As the food hopper was suspended above the cage floor, this prevented fouling of the food with urine or faeces, potentially adding to its mass artificially.

### 2.4.4.2 Meal Pattern Parameters

Raw data were analysed using previously published methods, considered suitable here, as they had been applied to data also generated by an outbred rat strain, with DIO induced by diets also formulated by Research Diets (Farley *et al*., 2003; Table 2.2).

**Table 2.2. Definitions of meal pattern parameters.**

| Parameter | Description |
|---|---|
| **Cumulative Energy Intake (kJ)** | Sum of food intake volume (expressed as energy content) across all meals in a defined time frame, such as or nocturnal or diurnal phase. |
| **Number of Meals** | Count of those meals which make up the cumulative energy intake. |
| **Meal Duration (min)** | Interval in minutes from the start to the end of a meal. |
| **Rate of Feeding (g/min)** | Volume of food intake consumed, expressed as grams per minute of meal duration. |
| **Inter-Meal Interval (min)** | Time interval from the end of one meal to the beginning of the next meal. |
| **Satiety Ratio (min/kJ)** | Ratio of inter-meal interval-to-meal size, expressed as energy content. |

Raw data collected by the LabMaster system was summarised according to these measures. Satiety ratio represented the duration of postprandial non-eating time per unit food intake. Source: Farley *et al.*, 2003. *Obes Res* **11**(7): 845- 851.

### 2.4.4.3 Syntax Development

In order to allow for systematic handling of the large quantities of raw data produced by the LabMaster system, and to aid in the calculation of meal pattern parameters, syntax script for Microsoft Excel 2007 was developed in-house (Table 2.3). Assistance in the development of this script was provided by Dr. A. Jones, leader of the Post-Genomic Bioinformatics Group, Institute of Integrative Biology, University of Liverpool.

**Table 2.3. Syntax script.**

| Function | Script |
|---|---|
| **Food** | =IF((D13-D12) >0.1, D13-D12, 0) |
| **Is Meal End** | =IF( AND(L12<>0, L13=0), "Meal End", "") |
| **Is IMI End** | =IF( AND(L12=0,L13<>0), "End IMI","") |
| **Temp IMI Length** | =IF(M12="Meal End",0,O11+10) |
| **Real IMI Length** | =IF(N12="End IMI", O12,"") |
| **Temp Meal Length** | =IF(N11="End IMI",0,Q10+10) |
| **Real Meal Length** | =IF(M12="Meal End",Q12,"") |

This enabled processing of extensive raw data generated by the LabMaster system, aiding in the calculation of meal pattern parameters defined in Table 2.2.

### 2.4.4.4 Analysing Circadian Rhythmicity

Biological function and behaviour, including eating, exhibit 24-hour patterns that are driven by an endogenous circadian clock mechanism (Duguay & Cermakian, 2009). Metabolism, associated with feeding, also follows a circadian pattern (Queiroz, 1974; Hillman, 1976; Ramsey *et al.,* 2007; Laposky *et al.,* 2008; Maury *et al.,* 2010). Altered circadian behaviour and sleep patterns are associated with increased hunger, decreased lipid and glucose metabolism and changes in satiety that often result in obesity and diabetes (Knutson & Van Cauter, 2008).

The parameters of feeding rhythm and pattern include the size and frequency of meals, as well as the time of eating during the day or night. Recently, consumption of a diet high in fat has been shown to alter the expected pattern of circadian rhythmicity in relation to energy intake in mice (Kohsaka *et al.,* 2007) and rats (Hariri & Thibault, 2011) and as this pattern had yet to be explored in relation to PUFA intake, it was included as part of meal pattern analysis in this project.

### 2.4.4.5 Data Binning

In order to graphically represent the circadian pattern of feeding in this project, the raw feeding data generated by LabMaster software was 'binned'. Data binning is a pre-processing technique which replaces outliers with a mean value, reducing the

overall effect of the results into a summary measure format. This involved two processes: Firstly, meals were averaged over the 24- or 72-hour time frame, and secondly, the data were compressed into 30-minute intervals or 'bins', as described previously (Cattone *et al.*, 2007; Hariri & Thibault, 2011). Once the data had been 'binned', they were then plotted to demonstrate the distribution of meals over time, something which the summary measures of meal pattern analysis (Table 2.2) do not show. The technique was modelled on that used by Hariri & Thibault (2011), as shown in Figure 2.8.

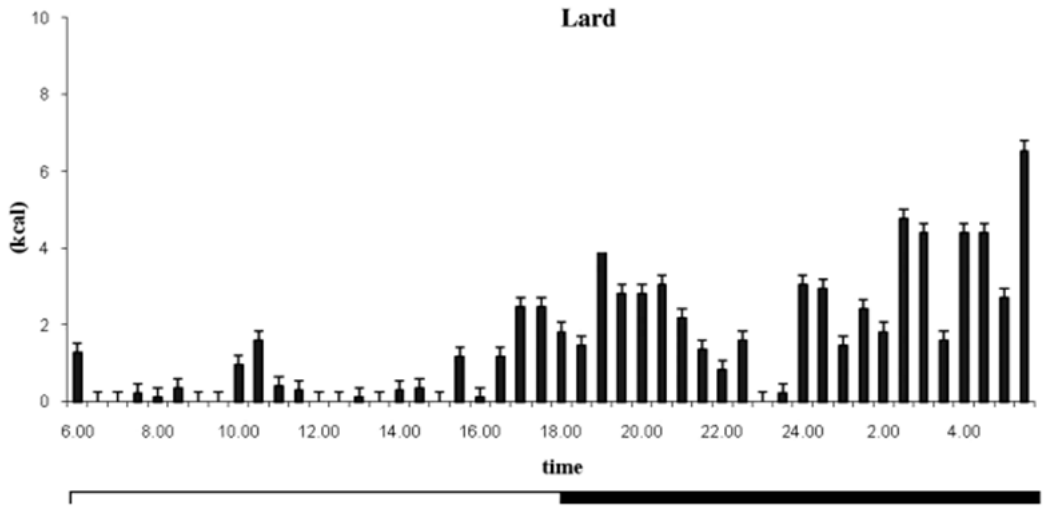



**Figure 2.8. Plot of circadian energy intake.** This graph displays energy consumed (as kcal, expressed as mean ± SEM) by rats during exposure to a high-fat, lard-based diet. Meals are indicated by vertical solid bars and diurnal and nocturnal phases by horizontal open and solid bars to the left and right, respectively, under the *x*-axis. Source: Hariri & Thibault, 2011. *Chronobiol Int* **28**(3): 216-228.

## 2.5 Termination and Surrogate Measures of Body Composition

Before termination, rats underwent an overnight fast to reduce variability in plasma concentrations of hormones and metabolites. The quantity of food given during this fast was approximately half of their nocturnal intake. The next day, under deep anaesthesia, rats underwent thoracotomy. A 1-2-ml blood sample was collected from the right atrium before transcardial perfusion with 50 ml of 10 mM PBS at room temperature, followed by 100 ml of fixative (4% paraformaldehyde, with 0.2% picric acid) at 4°C. During perfusion, the descending aorta was clamped to prevent fixation of abdominal organs, as only the brain required fixing for immunohistochemical purposes. After dissection, fixed brains were processed for histology, detailed in Section 2.10. White adipose tissue (WAT) depots (epididymal, perirenal and inguinal), interscapular brown adipose tissue (BAT), muscle (gastrocnemius and soleus), liver and spleen were also dissected free by blunt dissection, weighed, snap-frozen in liquid nitrogen, and stored at -80°C for later analysis. Specific anatomical sites of the WAT and BAT analysed can be seen in Figure 2.9. WAT and BAT were weighed as indices of adiposity and WAT further processed for molecular analysis, as described in Section 2.8. Muscle and spleen were processed for histological analysis, as detailed in Section 2.10.

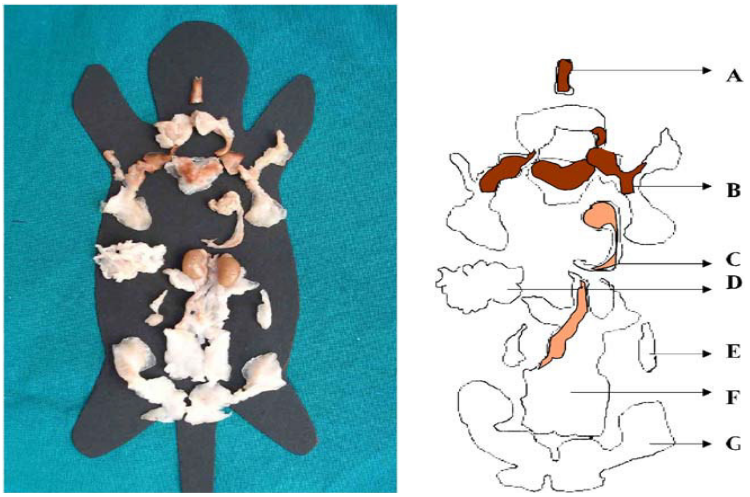



**Figure. 2.9. The adipose organs of a rodent.** The organ has been dissected with the aid of a surgical microscope and each depot has been placed on the diagram mimicking its anatomical position. The organ is made up of two subcutaneous and several visceral depots. **Abbreviations:** A= deep cervical; B= anterior subcutaneous (interscapular, subscapular, superficial cervical); C= visceral mediastinic; D= visceral mesenteric; E= visceral retroperitoneal (including epidiymal); F= visceral perirenal, G= posterior subcutaneous (dorso-lumbar, inguinal and gluteal). Source: Cinti *et al.,* 2005. *Prostaglandins Leukot Essent Fatty Acids* **73**(1): 9-15.

Assessment of body composition is an important indicator of nutritional status, and growth and development of individuals, as well as populations. Throughout these studies, fat mass was expressed as a percentage relative to body weight. A surrogate body composition measure of fat-to-lean tissue was calculated as the ratio of the sum of dissected WAT depot to muscle masses (Pickavance *et al.* 2001; 2005); i.e.

$$\text{Body Composition Index} = \frac{\Sigma \text{ WAT depot masses (g)}}{\Sigma \text{ muscle masses (g)}}$$

Animals predisposed to gaining weight are more 'food efficient', than their lean counterparts, meaning that rather than expending energy, they store it more readily as adipose tissue. Food efficiency was calculated as the ratio of cumulative body weight gain to 24-hour energy intake, over the course of diet exposure; i.e.

$$\text{Food Efficiency} = \frac{\Delta \text{ Body Weight (g)}}{\Sigma \text{ Energy Intake (MJ)}}$$

### 2.5.1 Ectopic Fat Deposition

Increased liver and BAT weights were taken as a possible indication of infiltration by white adipocytes. The former could also indicate metabolism of PUFAs, as discussed in Chapter 3, Section 3.4.1. Individuals who consume high-fat diets display variable responses in the way in which they store the fat within the body. Excess intra-abdominal (visceral) fat may be a marker of the inability of subcutaneous adipose tissue to act as an 'energy sink' for excess energy as a result of excess intake. The inability of subcutaneous fat to store excess energy may cause fat to accumulate at alternative locations such as the liver, skeletal muscle and heart. This process is known as ectopic fat deposition. Although liver fat has been frequently linked to excess intra-abdominal fat, several studies have shown that liver fat and intra-abdominal fat each contribute independently to the metabolic complications of abdominal obesity (Nguyen-Duy *et al.,* 2003). In order to monitor ectopic fat deposition, weights of liver and BAT were also recorded.

### 2.5.2 Bioimpedance Analysis

By the time work described in Chapter 5 was in the planning stages, facilities for assessment of whole-body composition had become available. It was measured by total-body electrical conductivity (TOBEC), using an Em-Scan/TOBEC® scanner designed for small animals (model SA-3000; Royem Scientific, Luton, UK). TOBEC is a non-invasive, non-destructive, rapid method for the *in vivo* estimation of lean body mass (LBM), total body fat (TBF), percent body fat relative to body weight (TBF%) and fat-free mass (FFM). Assessment of these measures was conducted using previously described equations (Morbach & Brans, 1992; Table 2.4).

**Table 2.4. Bioimpedance equations.**

| Body composition measures | Formulae |
|---|---|
| Lean body mass (LBM) | = 0.5E + (0.3 x total body weight) |
| Total body fat (TBF) | = total body weight – lean body mass |
| Total body fat % (TBF%) | = (total body fat x 100)/ total body weight |
| Fat-free mass (FFM) | = 16.28 + 0.4E |

These formulae were applied to the raw data collected in the study described in Chapter 5 only. E value was generated by the machine when scanning animals (see text). Source: Morbach & Brans, 1992. *J Pediatr Gastroenterol Nutr* **14**(3): 283-92.

This method involved measuring the TOBEC of rodents as they were passed through an electromagnetic field (Van Loan *et al.,* 1987), generating an E value, which was then substituted into the equations detailed in Table 2.4. The technique has been calibrated previously in-house for the measurement of body composition in male Wistar rats across a wide range of body weights (Cassidy *et al.,* unpublished observations).

## 2.6 Acquisition of Metabolic Data

### 2.6.1 Plasma Concentrations of Hormones and Lipids

Concentrations of appetite-related factors were measured to investigate any potential for their change over time, as a result of consuming diets high in fat. In order to do this, enzyme-linked immunosorbent and enzymatic assays were used. To characterise the models piloted in this project, a selection of 'classic' circulating factors relevant to appetite mediation (Chapters 3-5) and neurogenesis (Chapters 6 & 7) were assayed. Their significance in these contexts is outlined in the General Introduction (Chapter 1) and their relevance to high-fat consumption, in particular, is detailed further in individual experimental chapters.

### 2.6.2 Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assays (ELISAs) are analytical biochemical assays that employ a solid-phase enzyme immunoassay (EIA) to detect a substance in a liquid sample – in this instance, blood plasma. ELISAs employ a sensitive method, capable of detecting low concentrations of an antigen of interest, and because of this sensitivity, allow for the assay of small volumes, which is useful when samples are precious. The method requires an antigen, or target protein, which can be immobilized on the solid-phase and then bound to a detection agent, which is normally a specific antibody linked to an enzyme. Multiple wash steps ensure that only specific binding of the antibody takes place and a quantifiable signal is generated, most commonly detected as a colour change in the reaction mixture (Lequin, 2005).

### 2.6.3 Types of ELISA

There are several types of ELISA, including indirect, competitive and sandwich. Indirect ELISAs use non-specific antigen immobilisation methods so that small concentrations of antigen in plasma samples have to compete with other plasma proteins when binding to the well surface. The sandwich ELISA corrects this problem by employing a capture antibody specific for the test antigen. Competitive ELISAs are time consuming and require incubation with large sample volumes prior to the running of the assay. Since specific antigens were of interest and blood samples were precious in the studies presented here, sandwich ELISAs were used.

### 2.6.4 Principle of the Sandwich ELISA

In simple terms, a sandwich ELISA works on the principles illustrated in Figure 2.10, below.

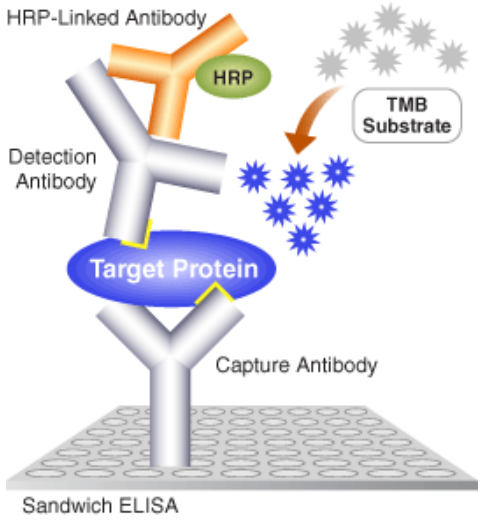



**Figure 2.10. Example of a generic sandwich ELISA.** The target protein first attaches to the plate coated with capture antibody. The detection antibody then attaches to the target protein, and the TMB substrate causes a reaction which results in a colour change that can be monitored using a plate reader. **Abbreviations:** HRP = horseradish peroxidase; TMB = 3,3',5,5'-tetramethylbenzidine. Source: www.sigmaaldrich.com.

For the ELISAs described here, the plate was coated with capture antibody and blocking agent, and the blood plasma was added to the wells, allowing the antigen of interest to bind to the capture antibody. Detection antibody was then added to the wells, where it bound to the antigen. Enzyme-linked secondary antibody was added, which, in turn, bound to the detection antibody, and the plate was washed so that any unbound reaction mixture was removed. Finally, a substrate was added which converted the enzyme present into a quantifiable colour change. The absorbency of the reaction mixture was then measured in the individual plate wells using a plate reader (Labsystems Multiskan Ascent, Thermo Fischer Scientific, Surrey, UK). This allowed for determination of the quantity of the antigen present in the blood samples, as the optical density (OD) of the individual samples was compared to those generated by known sample concentrations in a standard curve. All ELISAs were carried out according to the manufacturers' protocol.

### 2.6.5 ELISA Validation

Method validation, to check that the combined procedures of sample preparation and analysis yielded acceptably accurate, precise and reproducible results for a given antigen, had been tested by the manufacturers and documented in the product user's guide. A second validation was carried out in-house. This involved the assaying of standards and control samples of known concentration provided with the kit, as well as spare in-house samples, repeatedly over several days for all of the ELISAs used. This enabled the standardising of experimental conditions, setting up of parameters on the plate reader, and checking of results from assayed samples within the recommended range of the kit.

### 2.6.6 Mouse/Rat Leptin ELISA

Plasma leptin concentrations were measured using a commercially available kit (Peprotech, Inc., NJ, USA). It contained key components required for the quantitative measurement of natural and/or recombinant murine (m) leptin in a sandwich ELISA format. This involved the use of an antibody raised against mLeptin to capture the protein, and a biotinylated antigen-affinity purified anti-mLeptin antibody to detect it. An ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid] liquid substrate was used to monitor concentration of product measured as a colour change. The sensitivity range of the kit was 20-1250 pg/ml, and the cross reactivity was 100% with rat, and 2% with human, leptin. The samples were assayed undiluted at a volume of 50µl. At 1ng/ml the intra- and inter-assay coefficient of variation were 3.5% and 4.2% respectively (Peprotech, Inc., NJ, USA).

### 2.6.7 Rat Insulin ELISA

Plasma concentrations of insulin were measured using a commercially available kit (Mercodia, Uppsala, Sweden). Initially, an ELISA of standard sensitivity was used, but it was unable to detect insulin in the samples; therefore, an ultrasensitive rat insulin ELISA was used instead. During incubation, insulin in the sample reacted with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the plate. This kit was used in conjunction with control samples of rat insulin also supplied by the company [Diabetes Antigen Control (Low, Medium, and High)], as well as a TMB (3,3',5,5'-tetramethylbenzidine) liquid substrate to monitor

concentration of product measured as a colour change. The sensitivity range of the kit was 0.020-450 µg/L and the cross-reactivity was 100% with rat, 75% with mouse, and <0.05% with human insulin. The samples were assayed undiluted at a volume of 25µl. At 0.2ug/L the intra- and inter-assay coefficient of variation were 2.6% and 3.5% respectively (Mercodia, Uppsala, Sweden).

### 2.6.8 Rat Adiponectin ELISA

Plasma adiponectin concentrations were measured using a commercially available kit (ALPCO, NH, USA). Adiponectin is often referred to by molecular subtype (low, medium and high molecular weights), and this kit involved the use of specific, high-affinity antibodies to measure total adiponectin in the sample. It was selected because measurements of total adiponectin are of greater diagnostic value than any one of the subtypes alone (Blüher *et al.,* 2007). It has been demonstrated that total serum adiponectin is significantly decreased in insulin-resistant and type 2 diabetic, compared to insulin-sensitive subjects, and that this cannot be replicated when measuring subtypes alone (Lara-Castro *et al.,* 2006). Furthermore, studies have shown correlations between total serum adiponectin and insulin sensitivity and the ability of total serum adiponectin concentration to predict insulin resistance and impaired glucose tolerance (Blüher *et al.,* 2007). TMB liquid substrate was used to monitor concentration of product measured as a colour change. The sensitivity range of the kit was 0.25 - 10 ng/ml and the cross reactivity was 0% with mouse, rat, rabbit, goat, sheep, pig, horse and bovine samples of adiponectin. Prior to use, samples were diluted to 1:2,500 at a volume of 5µl. At 4µg/ml the intra- and inter-assay coefficient of variation were 4.5% and 5.0% respectively (ALPCO, NH, USA).

### 2.6.9 Rat BDNF ELISA

Plasma concentrations of BDNF were measured using a commercially available kit (Boster Biological Technology Ltd., Wuhan, China). TMB liquid substrate was used to monitor concentration of product measured as a colour change. The sensitivity range of the kit was 30-2000 pg/ml, and there was no detectable cross reactivity with any other cytokines. Prior to use, samples were diluted to 1:10 at a volume of 25µl. At 160pg/ml the intra- and inter-assay coefficient of variation were 5.6% and 4.2% respectively (Boster Biological Technology Ltd., Wuhan, China).

### 2.6.10 Rat/Mouse Corticosterone ELISA

Plasma concentrations of corticosterone were measured using a commercially available kit (Demeditec Diagnostics, GmbH, Germany). This ELISA was based on competitive binding principles; thus, an unknown amount of corticosterone in the sample, and a known amount of corticosterone conjugated to horseradish peroxidase, competed for binding sites of corticosterone antiserum coating the plate wells. This kit was used in conjunction with control samples of rat corticosterone also supplied by the company (Rat Control Set DEV99RC). TMB and hydrogen peroxidase liquid substrate were used to monitor concentration of product measured as a colour change. The sensitivity range of the kit was 3.6-245.5 ng/ml, and the cross reactivity was <0.02% with other steroids, including aldosterone, cortisol, deoxycorticosterone, estriol, estradiol, progesterone, testosterone and pregnolone. The samples were assayed undiluted at a volume of 25µl. At 180ng/ml the intra- and inter-assay coefficient of variation were 5.3% and 4.8% respectively (Demeditec Diagnostics, GmbH, Germany).

An illustration of how samples were typically arrayed for analysis in the current studies can be seen in Figure 2.11.


**Figure 2.11. Example ELISA plate design**. All plasma samples across the exposure period were counterbalanced according to dietary group. The standard curve was always on the left of the plate to prevent contamination of the samples with standard and control solutions during wash steps. **Abbreviations:** PUFA = polyunsaturated fatty acid; SFA = saturated fatty acid.

## 2.7 Enzymatic Assays

The enzymatic assay is a useful method for the quantitative determination of the concentration of a particular biological compound within a solution, often plasma or serum. It exploits the fact that the compound of interest will be converted, dependent on the presence of a certain enzyme or enzymes, into another compound through a series of reactions. This commonly produces a chromophore, such that the concentration of the compound of interest is directly related to the depth of colour in the solution and is thereby quantifiable at a particular wavelength using a spectrophotometer. Such assays are of obvious diagnostic value, and many have been conveniently developed to provide all required components of the enzymatic pathway in a single kit. This speeds up routine laboratory analyses significantly (Glick & Pasternak, 2003). The plasma samples assayed in this way were obtained as described above [Location 2.2].

### 2.7.1 Principle of the Triglyceride Assay

The commercial assay for the determination of triglyceride (TG) concentrations in plasma (Sigma-Aldrich Co., Ltd., Dorset, U.K.) involved a series of reactions, as follows:

TGs are enzymatically hydrolysed by lipoprotein lipase (LPL) to free fatty acids (FFAs) and glycerol:

$$\mathbf{TGs + H2O \xrightarrow{LPL} Glycerol + FFAs}$$

The glycerol is phosphorylated by ATP with glycerol kinase (GK) to produce glycerol-1-phosphate and ADP:

$$\mathbf{Glycerol + ATP \xrightarrow{GK} glycerol\text{-}1\text{-}phosphate + ADP}$$

Glycerol-1-phosphate is oxidised to dihydroxyacetone phosphate (DAP) by glycerol phosphate oxidase (GPO) producing hydrogen peroxidise ($H_2O_2$):

$$\mathbf{Glycerol\text{-}1\text{-}phosphate + O_2 \xrightarrow{GPO} DAP + H_2O_2}$$

In a colour reaction catalysed by peroxidase (POD), the $H_2O_2$ reacts with 4-aminoantipyrine (4-AAP) and sodium *N*-ethyl-*N*-(3-sulfopropyl) *m*-anisidine (ESPA)

to produce a red coloured quinoneimine dye that shows an absorbance maximum at 540 nm.

The increase in absorbance at 540 nm is directly proportional to the triglyceride concentration of the sample:

$$\mathbf{H_2O_2 + 4\text{-}AAP + ESPA \xrightarrow{POD} Quinoneimine\ dye + H_2O}$$

### 2.7.2 Protocol for Determination of TG Concentration in Plasma

Only a single reagent was required for this test, and its active ingredient included ATP, 4-AAP, ESPA, POD, GK, GPO and LPL. One ml of the reagent was added to plastic cuvettes containing plasma samples (25µl volume) and distilled water, in the case of the blank. The final concentrations of the above ingredients were 2.5 mM, 0.8 mM, 1 mM, 5.4 units per ml (U/ml), 0.56 U/ml, 6 U/ml and 31 U/ml, respectively. Samples were compared to an aqueous solution of glycerol standard (0.025 mg/ml; Sigma-Aldrich Co., Ltd., Poole, Dorset, U.K.) mixed with reagent. The solutions were mixed and after a reaction time of 15 min at room temperature, absorbances were read at 520 nm, referencing against the blank. These values were then applied to the following formula:

$$[\text{TG, mg/ml}] = \frac{\text{absorbance of sample}}{\text{absorbance of calibrator}} \times \text{concentration of standard}$$

where

concentration of standard = 0.26 mg/ml (equivalent triolein concentration).

## 2.8 RNA Extraction from White Adipose Tissue

### 2.8.1 TRI-Reagent Method

Extraction of ribonucleic acid (RNA) from epididymal, perirenal and inguinal white adipose tissue (WAT) depots was carried out using the TRI-reagent® method (Sigma Aldrich, Dorset, UK). In a chemical fume hood, forceps and homogenizer blade attachments were pre-treated with RNase AWAY™ (Molecular BioProd Inc, CA, USA) and rinsed with UV-irradiated distilled water several times prior to use, to avoid contamination. Between the processing of tissue samples from each dietary group, the homogenizer was again rinsed twice in UV-irradiated distilled water. Approximate 100-mg blocks of tissue were cut from the frozen sample, using a sterile scalpel blade and forceps, and placed into a 4-ml collection tube. One ml of TRI-Reagent® was added to the tube and the tissue homogenized using an electric homogenizer (Polytron Ultra-Turrax T25). The homogenate was then transferred into a 2-ml micro centrifuge tube and centrifuged at 12,000 rpm for 10 min at 4˚C. The clear supernatant layer was then transferred into a fresh 2-ml tube. After leaving the tissue to stand at room temperature for 5 min, 200 µl of chloroform (Fisher Scientific, Leicestershire, UK) were added. Each tube was then vortexed for 15 sec and allowed to stand for 10 min at room temperature. This was followed by centrifugation at 12,000 rpm for 15 min at 4˚C, separating the solution into three layers, a lower red phenol layer (protein), a white interphase layer (DNA) and a colourless upper layer (total RNA). The upper layer containing the RNA was transferred into a fresh 2-ml tube. To reduce contamination of the sample with DNA, 50 µl of isopropanol (Sigma-Aldrich, Dorset, UK) was added and the sample vortexed and allowed to stand for 10 min at room temperature before further centrifugation at 12,000 rpm for 10 min at 4˚C. The supernatant was then transferred into a 1.5-ml tube and 450 µl isopropanol added to precipitate the total RNA. This was followed by vortexing, incubation of the sample for 10 min at room temperature and centrifugation at 12,000 rpm for a further 10 min at 4˚C. On completion of this step, the supernatant was discarded, and 100 µl of a 75% solution of molecular grade ethanol (VWR International Ltd, Devon, UK) in ultra-pure water (0.1 µm filtered, DNase and RNase free) was added to the RNA pellet. This was followed by vortexing and centrifugation at 12,000 rpm for 5 min at 4˚C. The supernatant was again removed and the RNA pellet allowed to air dry for 5 min at room temperature.

The RNA pellet was then dissolved by adding 10 µl of ultra-pure water and briefly vortexed. Between extractions, equipment was cleaned of tissue, rinsed, decontaminated by soaking in 1% Virkon (Antec International, Suffolk, UK) for two hours and finally rinsed. RNA samples were stored at -80°C until use.

#### 2.8.1.1 RNA Quantification

Total RNA was diluted 1:70 by adding 1 µl to 69 µl of distilled water. The diluted sample was placed in a plastic cuvette, inserted into a spectrophotometer (BioPhotometer, Eppendorf UK Limited, Stevenage, UK) and light absorbance measured at 260 nm (A260) and 280 nm (A280). Distilled water was used as a blank. Maximal light absorbance by nucleic acids occurs at A260, whilst proteins absorb light maximally at A280 (Glasel, 1995). The extinction coefficient of RNA at A260 is approximately 40. The concentration of RNA in each sample was determined automatically by the Beer-Lambert law (Beer, 1852):

[RNA] µg/µl = A260 x 40 x DF,

where

Dilution Factor (DF) = 70

The concentration of protein in each sample was determined:

[Protein] µg/µl = A280 x 40 x DF,

where

DF = 70

The ratio between the values obtained at A260 and A280 indicated the purity of the RNA in the sample. A ratio of 1.8-2.0 suggested high purity. If samples were of low purity, then RNA extraction was repeated.

### 2.8.2 Real-Time Polymerase Chain Reaction (qPCR) – Taqman System

#### 2.8.2.1 Real-Time Polymerase Chain Reaction

Real-time PCR (qPCR) is a commonly used technique that allows the relative quantification of the amount of cDNA template in a sample. It monitors the amount of PCR product formed during each cycle *via* detection of a fluorescent signal (Logan *et al.,* 2009). The signal emitted increases in proportion to the amount produced. This is particularly evident during the exponential phase of the reaction (Glick & Pasternak, 2003). There are a number of different detection methods available for qPCR; these include TaqMan Probe Systems, SYBR Green I, Molecular Beacons and Hybridisation Probes (Tamarin, 1999). The in-house method of choice for qPCR detection was the TaqMan Probe System.

#### 2.8.2.2 Taqman® System

This system involved the use of forward and reverse primers and a TaqMan probe (Applied Biosystems, Cheshire, UK) to amplify the gene of interest. The TaqMan probe is an oligonucleotide with a fluorescent (reporter) dye (FAM-6-carboxyfluorescein) on the 5' base, and a quenching dye (TAMRA-6-carboxytetrametheyrhodamine) on the 3' base (Lawyer *et al.,* 1993). Energy is transferred from the reporter dye to the quenching dye by a process known as fluorescence resonance energy transfer (FRET). FRET is undetectable by the PCR machine, yet when the Taq polymerase copies a template onto which the probe is bound, its 5' exonuclease activity cleaves the probe separating the reporter and quenching dyes, ending the FRET (Giulietti *et al.*, 2001). The reporter dye is then able to emit fluorescence at a wavelength of 518 nm for detection of FAM, which the PCR machine can detect (see Figure 2.12). The amount of fluorescence increases in each cycle proportionally to the DNA amplification (Logan *et al.,* 2009; see Figure 2.13). This accumulation of product is detected by monitoring the increase in fluorescence of the reporter dye plotted after normalisation to a reference dye, in this instance ROX (carboxy-X-rhodamine). The reference dye is used to correct for differences in reaction volumes between wells (Sails, 2009).

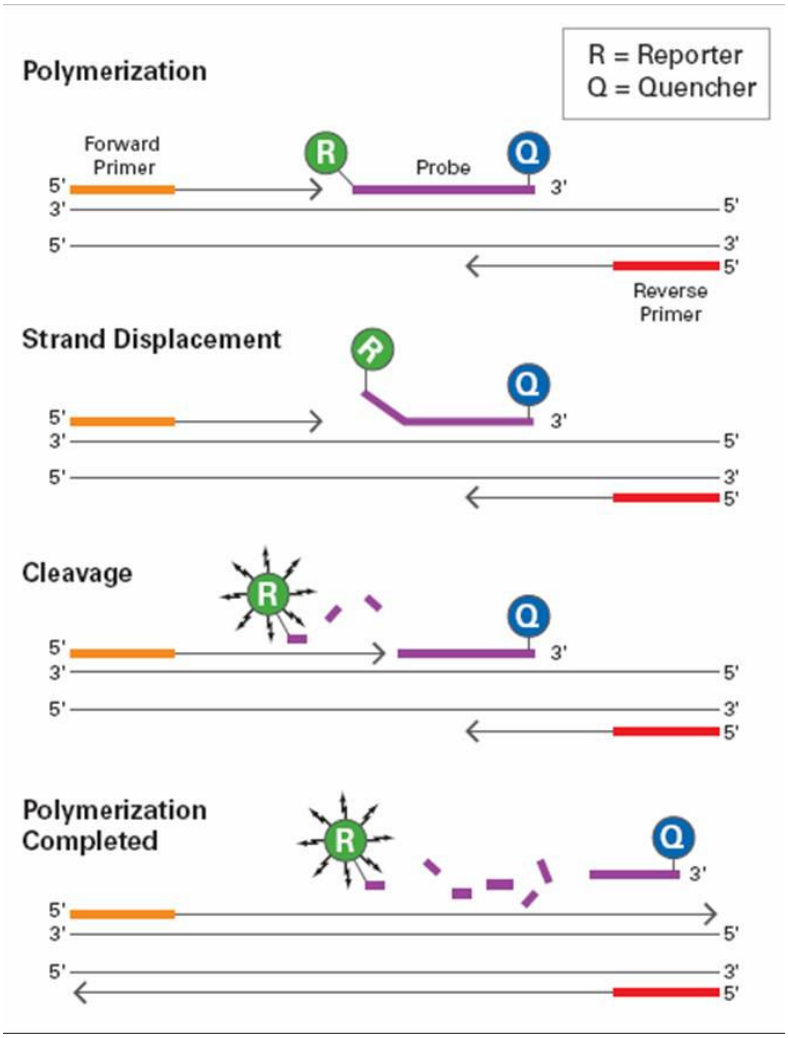



**Figure 2.12**. **TaqMan detection method for qPCR**. The TaqMan assay uses a probe oligonucleotide with a reporter fluorophore at the 5' end (R), and a quencher dye at the 3' end (Q). As the product is amplified, the 5'-to-3' exonuclease activity cleaves the end of the probe, allowing fluorescence emission from the reporter dye to occur. This emission is then monitored by the PCR machine. Source: www.appliedbiosystems.com.

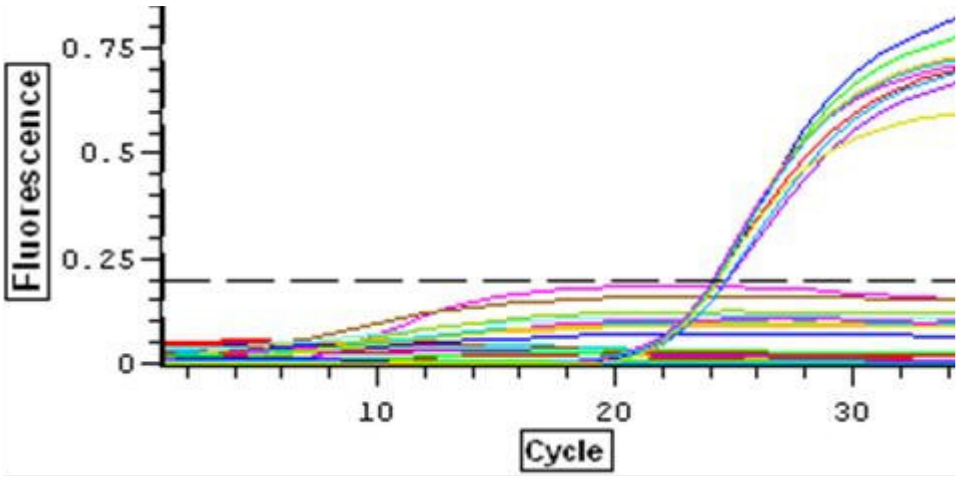



**Figure 2.13**. **Example plot demonstrating the amplification of fluorescent product produced at each cycle of PCR.** The amount of fluorescence increases in each cycle proportionally to the DNA amplification. The accumulation of product is then detected by monitoring the increase in fluorescence of the reporter dye plotted after normalisation to a reference dye. Source: www.bio-rad.com.

### 2.8.2.3 Preparation for Real-Time PCR

A master mix was prepared for each gene of interest, comprised of forward and reverse primers (see Table 2.5), Taqman probe and GoldStar enzyme (qPCR Core Kit RT-QP73-05; Eurogentec Ltd., Hampshire, UK), and according to the following protocol (per well): 1.25 µl 10x reaction buffer, 1.25 µl (2.5 mM) 50 mM magnesium chloride, 0.5 µl (100 mM) 5 mM dNTP, 0.038µl Sense primer, 0.038 µl anti-sense primer, 0.063 µl Taqman probe, 0.0625 µl (0.025 U/µl) of 5 U/µl Hot Goldstar enzyme and 8.29 µl Ultra-pure water, for a total reaction mixture of 11.5 µl per well.

The mix was aliquoted into a 96-well plate, and one µl of template cDNA sample was then added to each well for a total reaction volume of 12.5 µl. Non-template controls were included for all primers and probes. The plate was then sealed and centrifuged at 1000 rpm, for 15 sec (IEC Centra-7R Centrifuge, Damon, Global Medical Instrumentation Inc., Minnesota, USA).

**Table 2.5. Details of specific primers used for real-time qPCR analysis.**

| Primer | Product (bp) | Conc. (nM) | Sequence (5'-3') |
|---|---|---|---|
| **B-actin s** | 101 | 300 | GACAGGATGCAGAAGGAGATTACTG |
| **B-actin as** | | 300 | GAGCCACCAATCCACACAGA |
| **B-actin P** | | 225 | CACCATGAAGATCAAGATCATTGCTCCTCCT |
| **Leptin s** | 100 | 900 | AACCCTCATCAAGACCATTGTCA |
| **Leptin as** | | 900 | CCCGGGAATGAAGTCCAAA |
| **Leptin P** | | 225 | TGACATTTCACACACGCAGTCGGTATCC |
| **Adiponectin s** | 64 | 900 | CCCCTGGCAGGAAAGGA |
| **Adiponectin as** | | 900 | CCTACGCTGAATGCTGAGTGAT |
| **Adiponectin P** | | 225 | AGCCCGGAGAAGCCGCTTACATG |

The primers used for qPCR were β-actin, leptin and adiponectin (Eurogentec Ltd., Hampshire, UK). **Abbreviations**: as = antisense primer; P = probe; s = sense primer.

When quantifying the level of expression of a particular gene of interest, it is compared with that of a gene expressed at a concentration which is known to be stable; this is known as a 'housekeeping' gene. The selection of an appropriate housekeeping gene is critical, as it is used to assess the relative degrees of expression of the genes of interest. Common examples of housekeeping genes include β-actin, glyceraldehyde-3-phosphate (GAPDH), 18S rRNA and RNA polymerase IIa (RNAPolIIa). Studies examining gene expression in WAT have successfully used β-actin as the housekeeping gene (Gorzelniak *et al*., 2001; Eisele *et al*., 2005; Zhang *et al.,* 2005), and this, therefore, was the gene of choice for the assays described here. A second housekeeping gene, RNAPolIIa was also tested. However, expression of this gene was variable within the tissue samples and therefore, its use as a housekeeping gene was abandoned.

### 2.8.3.4 Real-Time PCR Set-up

Reactions were performed using a real-time PCR machine (Mx3000P, Agilent Technologies, CA, USA). The plate was inserted into the heat block and amplifications were performed as follows:

1. 50˚C for 2 min (activation of Hot Goldstar enzyme)

2. 95°C for 10 min (denaturation)

3. 35-40 cycles (dependent on the level of expression of gene)

    i. 95˚C for 15 sec (denaturation)

    ii. 60˚C for 60 sec (combined primer annealing and extension)

### 2.8.2.5 Analysis of qPCR Data

Once amplifications were complete, data were collected and analysed by software (MxPro-Mx3000P software, Agilent Technologies, Berkshire, UK), which also displayed amplification plots on a log scale. The threshold was adjusted to reduce background noise from the plot. Results were exported as Microsoft Excel files and gene expression analysed by relative quantification, by the 2-ΔΔCT method (Livak & Schmittgen, 2001). Samples were normalised to values for the housekeeping gene, β-actin, and results expressed as fold changes of threshold cycle (CT) values relative to the controls which in this instance was the SFA-fed group:

$\Delta CT = CT(PUFA) - CT(\beta\text{-actin})$

$\Delta\Delta CT = \text{Mean } \Delta CT(PUFA) - \text{Mean } \Delta CT(SFA)$

$\text{Fold Change} = 2^{-\Delta\Delta CT}$

## PART B: Neurogenic Methods

### 2.9 Neurogenic markers

Mapping cell fate was originally carried out to understand the origin of tissues in an adult organism by establishing the association between individual cells at one stage and their progeny at later stages of development (Gimlich & Jochen, 1985). The process of neurogenesis has three stages; the initial growth of a cell, known as proliferation, the migration of the cell to specific regions within the brain, and finally, differentiation into a mature neuron. As discussed in Chapter 1, this has been studied extensively in the hippocampus (Eriksson *et al.,* 1998; Balu & Lucki, 2009; Figure 2.14).

The original studies which investigated cell proliferation *in situ*, and determined the origin, migration and fate of neuronal cells in the central nervous system, all involved the use of autoradiography with tritiated ([$^3$H]) thymidine (Sidman *et al.,* 1959; Angevine, 1965; Schlessinger *et al.,* 1975; Crespo *et al.,* 1986). This method was also used to provide the first evidence that neurogenesis occurs within the SVZ and DG of the adult rodent brain (Altman & Das, 1965; Altman, 1969). The use of this radiolabeled substrate was reported to induce cell-cycle arrest, apoptosis and DNA damage within the cell (Hu *et al.,* 2002) and therefore, the development of new strategies to study cell proliferation and neurogenesis resulted in the replacement of [$^3$H]thymidine with the thymidine analogue, 5-bromo-2-deoxyuridine (BrdU) (Nowakowski & Hayes, 2000; Taupin, 2007).

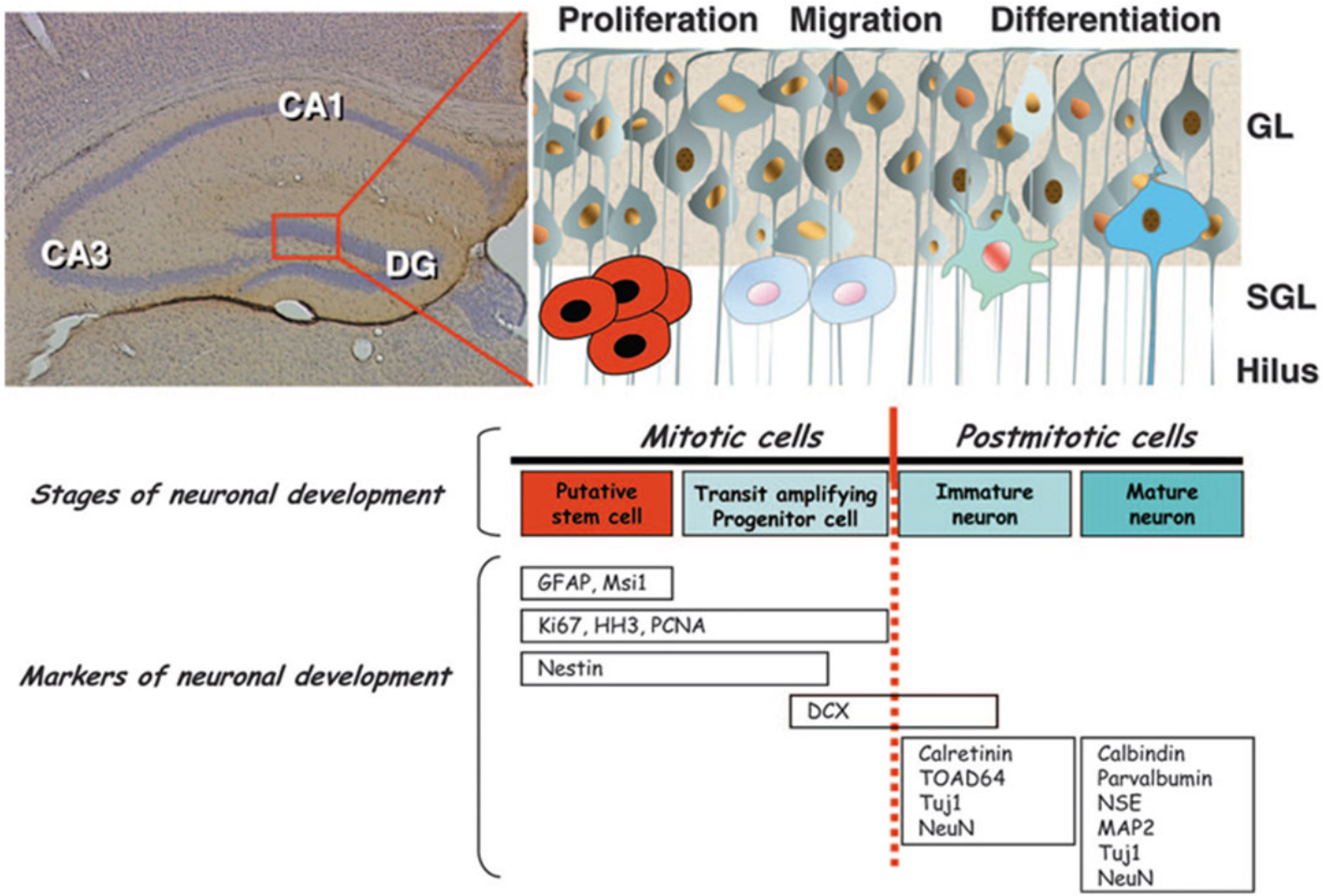



**Figure 2.14. Neurogenesis in the hippocampal system.** *Top panel:* a coronal section of rodent brain showing the sites of neurogenesis in the dentate gyrus (DG) of the hippocampus. Cells proliferate in the subgranular layer (SGL) located at the interface between the hilus and the granular layer (GL), where they migrate and differentiate into mature neurons. *Bottom panel*: the sequence of cell types involved in neuronal development, along with specific markers allowing cell identification (Kempermann *et al.*, 2004). Source: Abrous *et al.*, 2005. *Physiol Rev*. **85**(2):523-69.

### 2.9.1 Bromodeoxyuridine

Exogenous administration of BrdU is currently the most commonly used means of labelling the maximum number of proliferating cells in the brain. Although cell cycle proteins, such as Ki-67 and proliferating cell nuclear antigen (PCNA), can be immunostained in post-mortem brain sections, without the need for prior invasive technique, this provides only a "snapshot" of dividing cells at the point of animal sacrifice, and cannot indicate cell fate (Kee *et al.,* 2002; Gage *et al.,* 2008). [Immunostaining, or Immunohistochemistry, is discussed in Section 2.10]

BrdU is a halopyrimidine, a thymidine analog that intercalates into the DNA of dividing cells during the synthesis (S) phase of the cell cycle (Figure 2.15). It is used as a tracer for birth dating and monitoring of cell proliferation and subsequently, neurogenesis (Nowakowski *et al.,* 1989). BrdU is administered *in vivo* to an animal and the incorporated compound can then be detected *ex vivo* after termination by immunohistochemistry, using an antibody directed against single-stranded DNA containing BrdU (Gratzner, 1982). The process of neurogenesis is then confirmed using additional immunohistochemical markers of the type of cell or stage of development (Fig. 2.14).

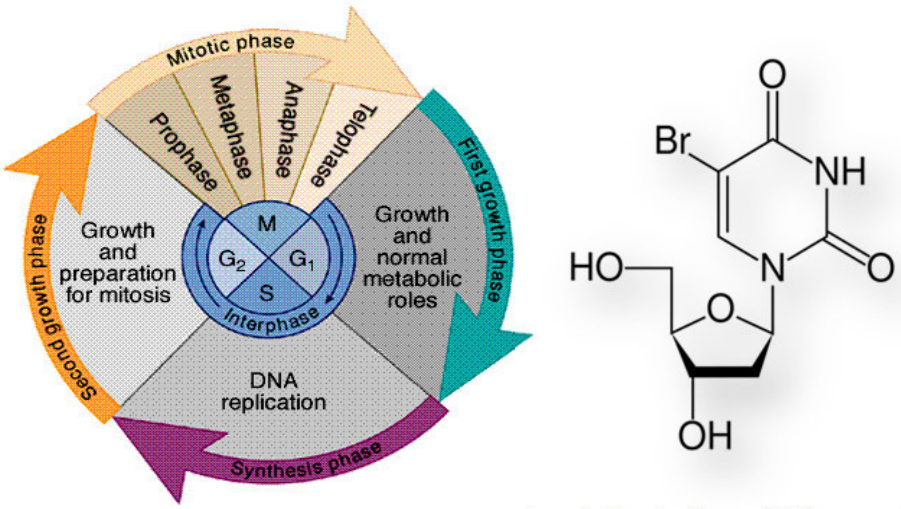



**Figure 2.15. Cell cycle diagram and structural illustration of 5-bromo-2-deoxyuridine (BrdU)** which incorporates into the cell cycle during the synthesis phase. BrdU is a thymidine analog that incorporates into DNA of dividing cells during the S-phase of the cell cycle, and is used for birth dating and monitoring cell proliferation. This can be revealed by immunohistochemistry using a monoclonal antibody directed against single-stranded DNA containing BrdU. BrdU immunohistochemistry is currently the most used technique for studying adult neurogenesis. **Abbreviations:** $G_1$ = first growth phase; $G_2$ = second growth phase; M = mitotic phase; S = synthesis phase. Sources: www.abcam.com and Taupin, 2007. *Brain Res Rev* **53**:198- 214.

Neural stem cells have the capability of either self renewing or becoming glial cells (astrocytes or oligodendrocytes) or neurons (Abrous *et al.,* 2005; Figure 2.16). Because of this potential, in instances where BrdU or other immunohistochemical markers are used to label cell proliferation, the techniques do not necessarily indicate neurogenesis. To confirm this process, markers of cell-type and stages of development must also be used (Fig. 2.14); for example, antibodies for the proteins doublecortin (DCX) and NeuN can both be used for immature and mature neurons, respectively. Furthermore, these immunohistochemical markers are successfully used to indicate cell proliferation in all tissues, not just neurons; therefore, they can be used to monitor processes such as myogenesis or adipogenesis (Kubis *et al.,* 2007).

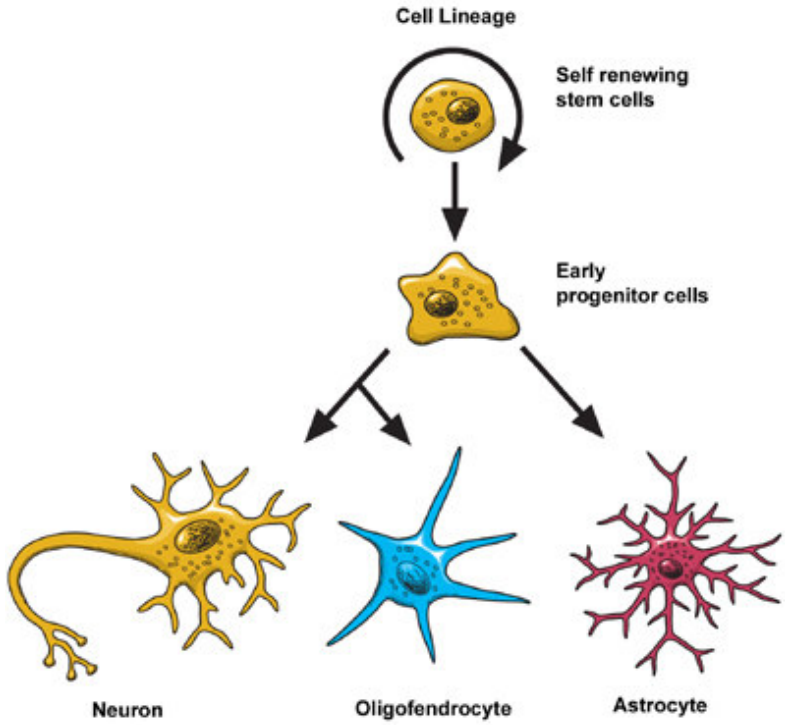



**Figure 2.16. Neural stem cell development.** Stem cells have the potential to self-renew or develop into early progenitor cells which then become neurons, oligodendrocytes or astrocytes. Source: www.prometheism.net; Abrous *et al.,* 2005. *Physiol Rev*. **85**(2):523-69.

### 2.9.2 Ki-67 & PCNA

Additional immunohistochemical markers which are used to detect cell proliferation include PCNA and Ki-67. PCNA is expressed during the S-phase of the cell cycle (Kurki *et al.,* 1986), as well as during DNA repair, and is also present in some non-

proliferating neurons (Ino & Chiba, 2000). This means that if PCNA is used as a marker of cell proliferation, the result may not be a true reflection of cell proliferation alone. In comparison, Ki-67 is expressed in all phases of the cell cycle, except the resting phase (Endl & Gerdes, 2000; Kee *et al.,* 2002; Zacchetti *et al.,* 2003) It has a short half-life and therefore, is not detectable during DNA repair (Scholzen & Gerdes, 2000; Zacchetti *et al.,* 2003). Hence, it is a more viable option for the labelling of proliferating cells. The quantification of Ki67-immunopositive cells has been shown to reflect proliferation in a manner consistent with BrdU labelling in the adult hippocampus, supporting the use of this marker for studying adult neurogenesis. Estimation of the extent of cell proliferation by quantification of Ki-67-positive cells in immunostained tissue is more accurate than that of BrdU- and PCNA-positive cells. As explained, this is because Ki-67 is expressed during most phases of the cell cycle, whereas BrdU and PCNA are markers of S-phase cells only. This, therefore, allows better estimation of proliferative activity alone (Kee *et al.,* 2002; Eadie *et al.,* 2005).

### 2.9.3 Technical Limitations of BrdU

Although BrdU labelling is currently the most utilised method for studying adult neurogenesis, there are known limitations to this technique. For instance, BrdU is a toxic substance, the integration of which alters DNA stability, increasing the risk of mutations, DNA strand breaks, and lengthening of the cell cycle (Saffhill & Ockey, 1985; Morris, 1991, 1992). Although no apparent toxic effect has been reported when studying adult neurogenesis specifically, it is likely that even low doses of BrdU may have a toxic effect on newly generated cells in the adult brain (Sekerkova *et al*., 2004), which may result in artificially low estimations of proliferation and increased measures of apoptosis (Gould & Gross, 2002).

DNA repair is a normally occurring process in the life of a cell and is carried out by cellular enzymes which secure genomic stability (Memo, 1999). Because DNA repair involves DNA synthesis and because BrdU is not a marker of cell proliferation *per se*, but DNA synthesis, there is a concern that BrdU immunohistochemistry may not only detect dividing cells in the brain, but also cells undergoing DNA repair (Taupin, 2007).

BrdU immunohistochemistry (IHC) has several limitations with respect to the study of cell proliferation and adult neurogenesis. This method involves the use of antibodies raised against DNA containing BrdU. In order for the antibody to gain access to the BrdU within the DNA, a denaturation process must be carried out (see Section 2.10.5). Common procedures involve the use of HCl acid treatments to hydrolyse the tissue (Moran *et al.,* 1985; del Rio & Soriano, 1989; Taupin, 2007). These sorts of harsh treatments affect tissue structure and antigenicity recognition in multiple antibody labelling studies, ultimately limiting identification of newly forming cells. Denaturation procedures further prevent the use of fluorescent counterstains, such as 4',6-diamidine-2'-phenylindole (DAPI; detailed below in Section 2.10.6) and propidium iodide (PI), both of which bind to double-stranded DNA and aid in the observation of cell nuclei (Chazotte, 2011).

Furthermore, the extent and intensity of BrdU immunostaining is largely dependent on the particular techniques used for detection and may not realistically reflect the amount of DNA replication (Nowakowski & Hayes, 2000). Therefore, data must be carefully interpreted and sequential immunostaining in adjacent tissue sections should be carried out with other markers of neurogenesis. Appropriate controls must also be used to ensure that incorporation of BrdU truly reflects the generation of new neurons in the adult brain (Taupin, 2007). Examples of these are outlined in Section 2.10.3.1.

### 2.9.4 Alternative Methods

Recently, the thymidine analogue, 5-ethynyl-2′-deoxyuridine (EdU), has also been investigated for detecting DNA synthesis (Cappella *et al.,* 2008; Chehrehasa *et al*., 2009; Zeng *et al.,* 2010). EdU is incorporated into the DNA of a cell during the replication stage of the cell cycle and is detected through a copper-catalysed reaction between the ethynyl group and fluorescent azide (Click-iTTM EdU Cell Proliferation Assay Kit; Invitrogen, Paisley, UK; Chehrehasa *et al.,* 2009). Because of the small size of the fluorescent azide, it can diffuse into the double-stranded DNA, eliminating the need for DNA denaturation and protecting the integrity and antigenicity of the sample. This new technique has been evaluated against the traditional BrdU method and has demonstrated comparable results. In some instances, EdU has proven to be more sensitive than BrdU. For example, in studies

where mice were intentionally stressed by physical restraint, counts for EdU positive immunoreactivity were drastically reduced below that of BrdU counts, suggesting, that as stress is known to reduce cell proliferation within the brain, this marker was more sensitive. While this new compound is currently under scrutiny, it has been suggested that the combined staining of both markers might provide a valuable double-labelling approach (Zeng *et al.,* 2010).

As this method is currently under development and extremely expensive to carry out, as well as for the reasons discussed above, a combination of approaches involving the use of BrdU and other *ex vivo* immunohistochemical markers was considered appropriate for the current project.

### 2.9.5 Modes of Administration of BrdU

BrdU must be administered *in vivo* to allow for incorporation into the DNA of cells. The compound is purchased as a powder, which is then dissolved in solution and administered either peripherally *via* intraperitoneal (i.p.) injections or orally, through the drinking water (Taupin, 2007), or centrally *via* intracerebroventricular (i.c.v.) infusion (Kokoeva *et al.,* 2005; Table 2.6). Most studies have involved repeated injection of BrdU i.p., at a recommended dosage and frequency (Table 2.7), to ensure a degree of uptake that will label the most dividing cells. This is a popular method because it avoids surgical intervention (Gould *et al.,* 2001; Kornack & Rakic, 2001a). However, as BrdU in human plasma is metabolised rapidly by dehalogenation, with a half life of approximately 10 minutes (Kriss *et al.,* 1963), and as it has to enter the circulation and cross the blood-brain-barrier (BBB), the concentration that reaches the brain, at least at certain sites, is often minimal in comparison to the initial administered dose (Kokoeva *et al*., 2007; Cifuentes *et al.,* 2011).

**Table 2.6. Routes of delivery, common concentration ranges and frequencies of delivery for BrdU administration.**

| Route of Delivery | Concentration | Frequency |
|---|---|---|
| i.p. injection | 50-100 mg/kg | 2-4 per day |
| i.c.v. infusion | 1 μg/μl | constant |
| drinking water | 1 mg/ml | constant |

Researchers have assessed the suitability of central BrdU delivery to better capture the proliferative potency of brain regions distant from the principal neurogenic sites, such as the hypothalamus and substantia nigra. They have found the number of labelled newborn cells to be much higher after i.c.v. infusion of BrdU than after i.p. delivery (Zhao *et al*., 2003; Kokoeva *et al*., 2007; Cifuentes *et al.*, 2011). Extended BrdU exposure methods, such as i.c.v. infusion and incorporation into drinking water are often required when studying adult brain sites with a generally low mitogenic activity (Zhao *et al*., 2003; Kokoeva *et al*., 2005, 2007; Bennett *et al.,* 2009; Cifuentes *et al.*, 2011). However, oral delivery may be preferred over i.c.v. infusion, as the invasive nature of the latter may induce stress, which is known to affect the rate of neurogenesis (Gould *et al.,* 1998). Despite the comparative ease of this method, and therefore, its compatibility with other study interventions which may run concurrently, it appears to have been used only occasionally (Zhao *et al*., 2003; Bennett *et al.,* 2009). It may be that researchers prefer to be able to control the amount of BrdU absorbed from drinking water (Zhao *et al.,* 2003): Animals in a given study will drink variable amounts, thereby receiving variable 'doses' of BrdU. In addition, it is uncertain how much intact BrdU crosses the gut lining, also potentially affecting the 'dose' which crosses the BBB. On the other hand this loss may be offset by the frequency of constant administration through drinking, compared to the intermittent delivery with i.p. administration. These issues are only speculative at present, as it appears that they have not been put to the test in any published papers in the field to date. In pilot studies undertaken during my MRes, I had directly compared all three routes of BrdU delivery in rat and found administration through the drinking water to be superior to i.p. injection and comparable with direct i.c.v. infusion, with respect to the extent of BrdU uptake in the hypothalamus, as indicated by the number of BrdU-immunolabelled cells. Therefore, this was the method of choice for the current project. The technicalities surrounding the stimulation and observation of cell proliferation in the adult rat hypothalamus using this method ultimately required study in their own right and are detailed in Chapters 6 and 7.

## 2.10 Immunohistochemistry

Immunohistochemistry (IHC) is a technique commonly used to investigate the localisation and distribution of a specific antigen, such as a protein, within a cell or tissue. In comparison, immunocytochemistry (ICC) is performed on samples of intact cells that have had their surrounding extracellular matrix removed, such as cells in culture, aspirates and blood smears (Polak & Norden, 2005). Accurate preparation of the sample for IHC is important for maintaining the original morphology of the tissue. This involves careful collection of the tissue, fixation and sectioning. Fixation is commonly carried out using a paraformaldehyde-based solution, and then the sample is sliced into sections using a microtome or cryostat. These sections can then be mounted onto slides or processed 'free-floating'. Sometimes the tissue sample may require additional processes to allow antibodies access to the antigen of interest. These steps may include antigen retrieval methods, such as acid denaturation (Gratzner, 1982; Moran *et al.,* 1985), described below with reference to BrdU-immunostaining (Section 2.10.5).

IHC employs antibodies which bind to the specific antigen in the tissue. Observation of this interaction can be achieved in several ways. For example, within the studies documented here, the antibodies had been tagged with a fluorophore and detected with an epi-fluorescent microscope. IHC is a popular technique in the field of neurobiology, as it enables examination of protein expression within specific structures of the brain, which are non-uniform, preventing the loss of information which occurs with techniques requiring tissue homogenisation. Its main disadvantage is that, unlike other techniques, such as Western blotting, where staining is commonly checked against a molecular-weight ladder, it is not possible to show that the staining observed corresponds to the protein of interest (Polak & Norden, 2005). For this reason, antibodies are thoroughly validated upon development using Western blotting; if the antibody is specific for the selected target, a band at the predetermined molecular weight for the target antigen will appear (Major *et al*., 2006; Bordeaux *et al.,* 2010). An alternative, but less commonly used, validation method involves the use of blocking peptides which have the same sequence used to create the antibody and are incubated with the antibody in excess. The antibody, with and without the blocking peptide, is then used to stain tissue samples which are known to express the target of interest. If the antibody is specific, the addition of the peptide will result in

no observed staining in the tissue (Skliris *et al.,* 2009). Although this method shows that the antibody is specific, it does not prove selectivity, as non-specific binding will also be inhibited by pre-adsorption with the peptide (Michel *et al.,* 2009). Therefore, the antibody in question should always be validated in-house with appropriate controls. These are defined below in Section 2.10.3.

### 2.10.1 Indirect Immunofluorescence

There are two methods involving the use of immunofluorescence, direct and indirect. Direct immunofluorescence involves the use of a primary antibody tagged with fluorophore. Although it involves fewer steps and is, therefore, less time-consuming than indirect immunofluorescence, it is not commonly used because the number of fluorescent molecules that can be bound to the primary antibody is limited and as a result the method is less sensitive and therefore, gives less signal amplification (Polak & Norden, 2005; see Figure 2.17).

Indirect immunofluorescence involves the use of two antibodies, an unlabeled primary antibody, raised in a host species, against the antigen of interest, and which specifically binds to the target antigen; and a secondary antibody, which carries a fluorophore, recognises the primary antibody, binds to it, and fluoresces. In this instance, multiple secondary antibodies can bind to a single primary antibody; therefore, providing greater signal amplification by increasing the number of fluorophore molecules per antigen (Polak & Norden, 2005; see Figure 2.17). Localisation of antigens in studies documented here was determined by indirect immunofluorescence.

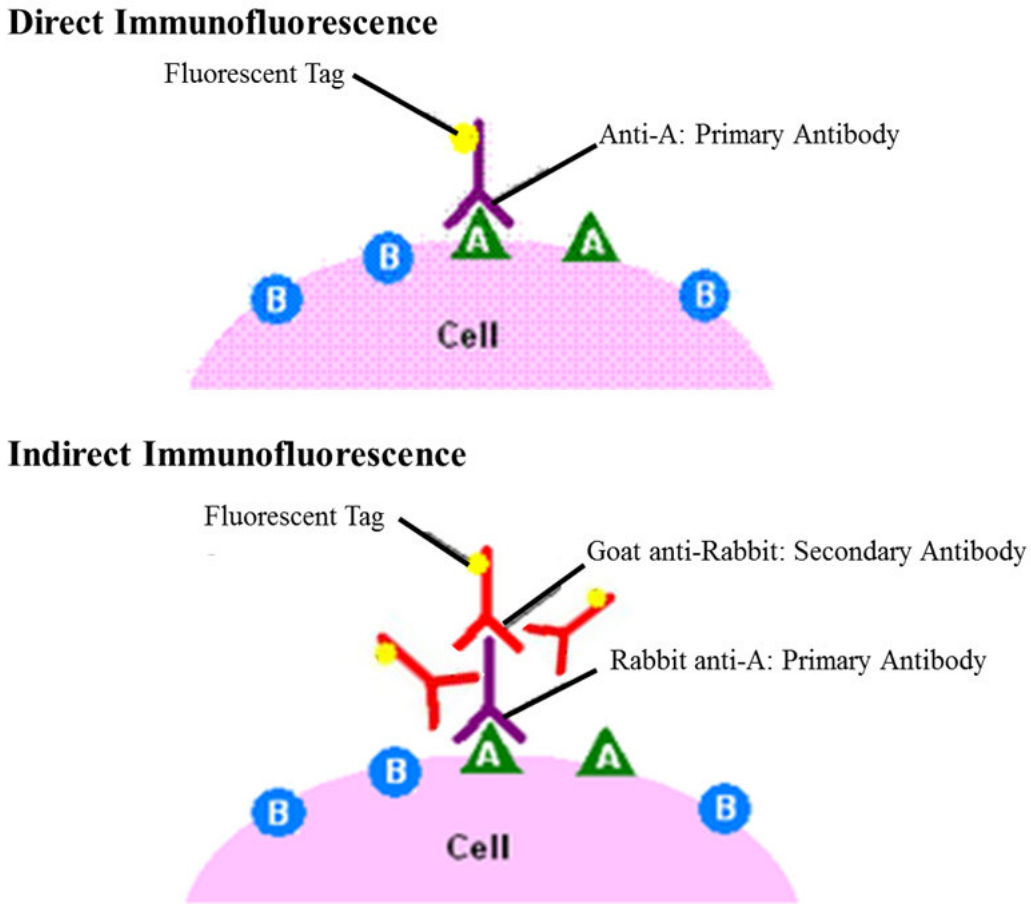



**Figure 2.17. Direct and indirect methods of immunofluorescent staining.** Direct method: Primary antibody with fluorescent tag binds directly to antigen of interest. Indirect method: Primary antibody binds to antigen of interest, secondary antibody with fluorescent tag binds to primary antibody. **Abbreviations:** A = target antigen; B = other antigen present on cell. Source: adapted from www.badrilla.com, using principles described in Polak & Norden, 2005. *Introduction to Immunocytochemistry*. 3rd Edition. BIOS.

### 2.10.2 Antibody Types

There are two different types of antibodies that can be used for indirect immunofluorescence, monoclonal and polyclonal. Monoclonal antibodies are specific to a single epitope (the part of the antibody which is recognised by antibodies in the immune system) and are therefore more specific (Polak & Norden, 2005). Monoclonal antibodies are produced in mice challenged with the specific antigen, and activated B-lymphocytes from the spleen, the source of the antibodies, are fused with cultured myeloma cells from mice of the same strain which are unable to produce the antibody. The fusion results in hybrid cells which continue to grow and divide in culture and also produce antibodies. One hybrid cell produces only one type of antibody. The cultured hybrid myeloma cells are gradually cloned by testing

the culture medium for secreted antibodies at each stage of culture. This leads to the development of a cell line derived from a single cell which produces a single antibody (monoclonal) (Figure 2.18; Ritter, 1986; Ritter & Ladyman, 1995). Monoclonal antibodies raised against the cell proliferation markers BrdU and PCNA were used in this project (Table 2.8; Chapters 6 & 7).

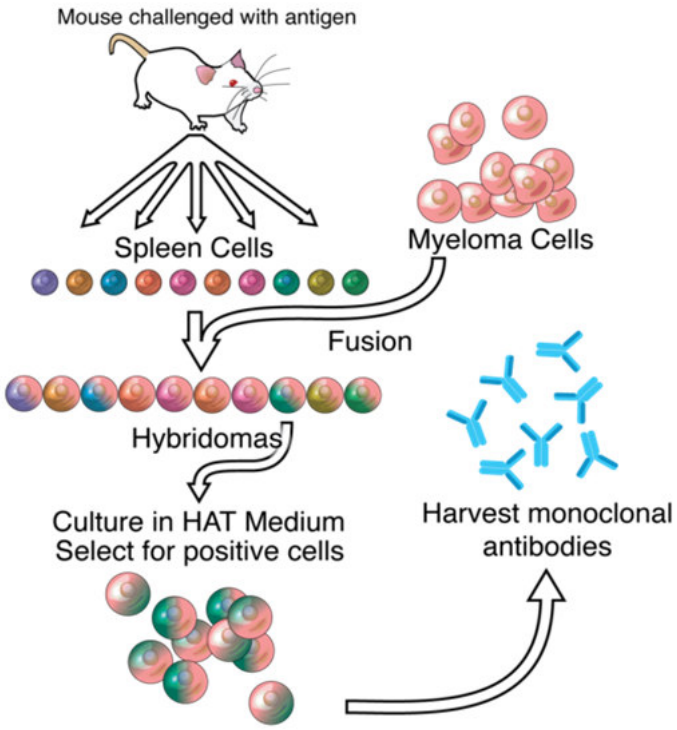



**Figure 2.18. Production of monoclonal antibodies.** Spleen cells from a mouse challenged with antigen are fused with myeloma cells, producing hybridomas. These are cultured in HAT medium (containing hypoxanthine, aminopterin, and thymidine), and monoclonal antibodies are harvested. Source: www.proteopedia.org based on principles described in Ritter & Ladyman, 1995 *Monoclonal Antibodies, Production, Engineering and Clinical Application*. Cambridge University Press, Cambridge.

In contrast, polyclonal antibodies are isolated from the host serum (Polak & Norden, 2005). Injection of the host animal with the antigen of interest induces an immune response specific to that antigen. This produces IgG immunoglobulins (Johnstone & Thorpe, 1996), which are then removed by purification of the host animal's serum (Figure 2.19).

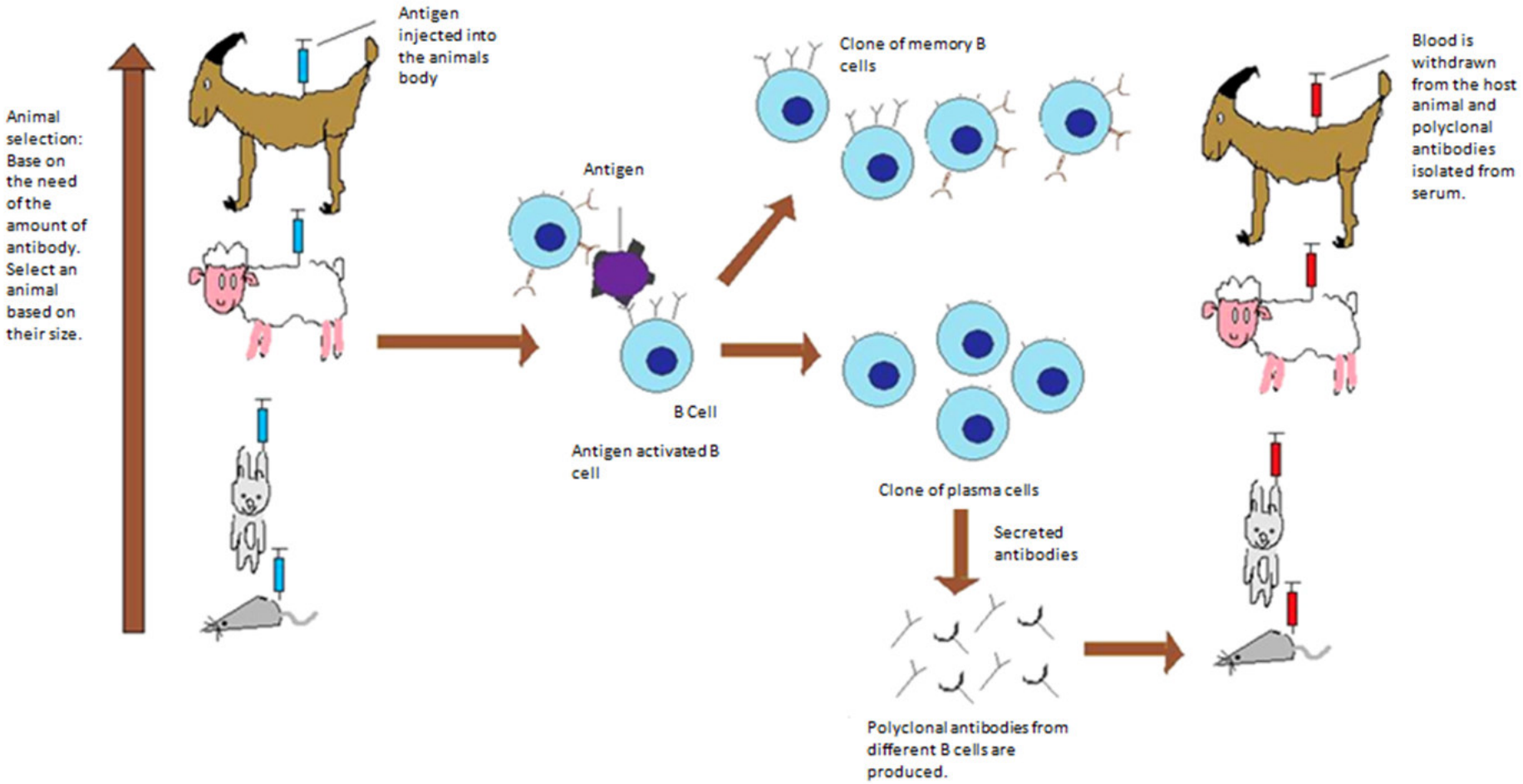



**Figure 2.19. Production of polyclonal antibodies**. The host species is selected based on quantity of antibody needed. Antigen is injected into the body of the animal, stimulating an immune response. Polyclonal antibodies from different B cells are produced and removed from the serum. Source: www.wikibooks.org based on principles described in Elsadig & Ali, 2012. *Polyclonal Antibody Production*. LAP LAMBERT Academic Publishing.

For detection purposes, antibodies are categorised as primary and secondary. Primary antibodies are raised against the antigen of interest, while secondary antibodies are raised against the immunoglobulins of the species used to raise the primary antibody. As mentioned previously, it is the secondary antibody that is directly bound to the fluorophore, which acts as a reporter molecule, allowing for detection of an antigen (Polak & Norden, 2005).

### 2.10.3 Controls for Immunohistochemistry

Sometimes antibodies bind to tissue sites that do not contain the specific antigen of interest; this is because the antiserum contains a mixture of antibodies including some from the host animals' serum. Their detection produces 'background' staining. Background staining is an issue, as it may mask staining of the antigen of interest preventing quantification. Background staining can also be caused by cross-reactivity between antibodies raised in varying species. Because of the similarities of immunoglobulin molecules from different species, it is possible that the secondary-layer anti-immunoglobulin will react with the primary-immunoglobulin of the animal in which the antigen is to be immunostained, for example anti-mouse immunoglobulin with rat immunoglobulin (Polak & Noorden, 2005). Most manufacturers test commercially available antibodies for cross-reactivity during development using ELISAs. Product data sheets will then specify any known cross-reactivity and the species which the antibody has been tested against.

There are two main types of controls, positive and negative, which aid in the confirmation of background staining and accuracy of results found.

#### 2.10.3.1 Positive Controls

Positive control samples known to contain the antigens in question can be obtained, often by harvesting appropriate tissue samples at the time of perfuse-fixation. For example, in the studies outlined here, spleen samples were removed during dissection and sections processed and stained alongside brain sections. Spleen was selected as a control tissue for the staining of proliferating cells, as it rapidly regenerates when injured, but also has a high turnover of cells in general (Levy *et al.,* 1976). The *pia mater* was also used, as an internal brain control, albeit outside the BBB. It is the innermost layer of the meninges, the membrane which surrounds the brain and is

lined by epithelial cells, a cell-type which self-regenerates (Decimo *et al.*, 2012). This rapid cell turnover is ideal for demonstrating uptake of BrdU. Without the use of such controls, a negative result on the test material cannot be confirmed as either real or artefact; because there is no guarantee that the reagents are in good working condition and have been applied in the correct order and at the correct dilutions. If the positive control is satisfactory, it is a reasonable assumption that the correct method has been carried out on the test material as well (Heyderman, 1972; Polak & Noorden, 2005).

#### 2.10.3.2 Negative Controls

Negative controls are preparations wherein application of the primary antibody solution is omitted. In all other respects, the treatment is exactly the same as for the test preparation. There should be no staining visible at the end of the reaction. If there is, it must be assumed that it is non-specific, and attempts to prevent it can be made. There are two commonly used solutions to this problem. The first is to dilute the antibody as much as possible prior to use, and the second is to block any potential binding sites with a highly concentrated protein solution. Either of these steps should lessen the effect of background staining mentioned previously. Non-specific binding sites can normally be blocked with normal serum or an 'inert' protein such as bovine serum albumin (BSA) or casein, at a concentration of 2% solution in buffer (Polak & Norden, 2005). However, these steps can generally be prevented by suitable wash steps, as these non-specific bonds are weaker than the specific ones (Buffa *et al.*, 1979). Thus, in the studies documented here, working antibody solutions were made from stock solutions containing 0.1% BSA and removed from sections by washing in 10 mM PBS multiple times between incubations (see Section 2.10.4, below).

#### 2.10.3.3 Absorption Controls

To demonstrate that an antibody is binding specifically to the antigen of interest, it can be pre-incubated with the same peptide (immunogen) used to immunise the animals that produced the antibody. This should deactivate the antibody, and the tissue should show little or no staining. This is often referred to as an absorption control or specificity control, and the peptide is often referred to as the blocking peptide (see Figure 2.20). The pre-absorbed antibody can then be incubated with tissue in place of the primary antibody alone. The staining pattern produced by the

primary antibody can be compared to that produced by the pre-absorbed antibody (Polak & Norden, 2005).

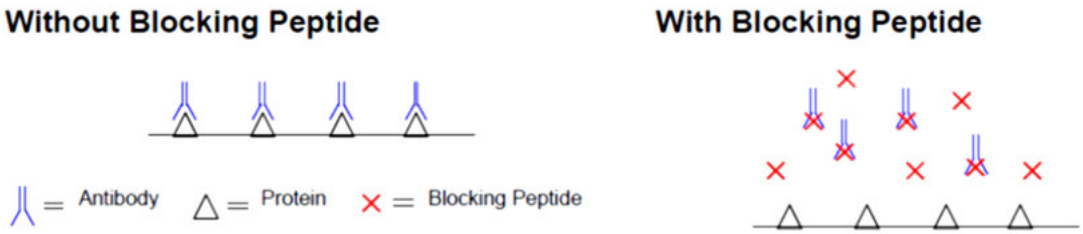



**Figure 2.20**. **Absorption Control.** As in a competitive assay, the immunogen peptide competes with the protein of interest in the tissue sample for antibody binding sites. Source: www.neuromics.com.

Absorption controls work best when the immunogens are peptides. However, if antibodies have been raised against a whole protein, then addition of the mixture of antibody and protein may result in even greater non-specific staining. Therefore, it is important to note that an absorption control using whole protein may not always confirm the specificity of an antibody for the protein of interest in the tissue. An absorption control is essentially a competitive assay, as the peptide competes with the protein of interest for antibody binding sites in the tissue sample (Figure 2.20). Therefore, absorption with excess peptide is preferable, to favour the antibody binding with the peptide while inhibiting binding with the protein of interest.

Both primary and secondary antibodies used in these studies had been well-characterised previously by the manufacturers, ourselves and others (as revealed by consultation with the literature) and were known to stain their target proteins without the need for additional specificity tests.

### 2.10.4 Immunohistochemistry and Image Analysis

For the studies detailed in this thesis, rats were perfuse-fixed (see Section 2.5) and brains were removed, placed in a brain blocker (Kopf®, David Kopf Instruments, CA, USA) and the hypothalamus blocked at the anterior-posterior coordinates of 8.16 and 3.36 mm (Paxinos & Watson, 2004), using the plane of the interaural line as reference. They were then post-fixed in the same fixative used for perfusion (4% paraformaldehyde, with 0.2% picric acid in 100 mM PB) for three hours and

transferred to cryoprotectant (30% sucrose in 100 mM PB) overnight at 4°C. Cryoprotectant was then refreshed, and brains stored until use.

Fixed hypothalamic blocks and positive control tissues were prepared as described above (Section 2.5). They were serially sectioned at a thickness of 50 µm, using a freezing microtome (Leica 2000R). Sections were collected free-floating into well-plates containing 10mM PBS. Any spare sections were stored at -20°C in cryoprotectant (50% v/v 10mM PBS; 30% w/v Sucrose; 1% w/v Polyvinylpyrrolidone; 30% v/v Ethyleneglycol). Sections were washed 3 x 10 minutes in 10 mM PBS between each of the following steps:

Sections were first incubated in primary antibody solution overnight (15-20 hrs) at 4°C. This was followed by a 1-hour room temperature incubation in secondary antibody solution in the dark. Both incubations were carried out under constant agitation on an agitator (200 Double Rocker, VWR International Ltd., Leicestershire, UK). Details of primary and secondary antibodies can be seen in Table 2.7. Optimal working concentrations had been determined in earlier tests on spare tissue through by applying serial dilutions prepared with 10mM PBS containing 0.3% Triton-X, a detergent to aid penetration of the antibody into the tissue.

**Table 2.7. Details of primary and secondary antibodies.**

| Antigen | Host | Dilution | Manufacturer | Secondary | Dilution |
|---|---|---|---|---|---|
| BrdU | Mouse | 1:50 | Roche | Mouse Dylight 594 | 1:500 |
| Ki-67 | Rabbit | 1:100 | Vector Labs | Rabbit Dylight 594 | 1:500 |
| PCNA | Mouse | 1:100 | Santa Cruz | Mouse Dylight 594 | 1:500 |
| A4.74 | Human | 1:10 | DSHB | Mouse Dylight 594 | 1:500 |
| A4.840 | Human | 1:10 | DSHB | Mouse Dylight 594 | 1:500 |

Antibodies were used in studies described in Chapters 4, 6 and 7, as markers of cell proliferation (BrdU, Ki-67 and PCNA) and muscle fibre-type (A4.74 and A4.840). Secondary antibodies were manufactured by Jackson ImmunoResearch Laboratories (Stratech, Suffolk, UK). Dylight 594 is a red fluorophore-conjugate. **Abbreviations:** A4.74 = fast fibre type, A4.840 = slow fibre type, BrdU = bromodeoxyuridine, PCNA = proliferating cell nuclear antigen.

### 2.10.5 DNA Denaturation

Protocols to detect BrdU by IHC have been devised using monoclonal antibodies directed against single-stranded DNA containing BrdU (Gratzner, 1982). This requires the denaturation of DNA, the standard procedures for which involve partial hydrolysis of the tissue with HCl acid (Gratzner, 1982; Moran *et al.,* 1985). Prior to immunolabelling with anti-BrdU antibodies, brain and spleen sections were incubated in 1M HCl for 30 minutes at 37°C (Chapters 6 & 7), and muscle sections were incubated in 1 M HCl for 15 minutes at room temperature (Chapter 4). The acid was then neutralised by rinsing the sections three times in 10 mM PBS with constant agitation. Methods described by Wojtowicz & Kee (2006) were adapted and optimised to suit the tissue-types processed here; these modifications included increased duration of wash steps, reduced temperature for denaturation and reduced time for acid exposure for the thinner muscle sections (10- *vs.* 50-µm-thick).

### 2.10.6 DAPI

DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich Co., Ltd., Dorset, U.K.), a fluorescent molecule that binds strongly to DNA, was applied to muscle sections (1% solution) described in Chapter 4 to act as a counterstain for BrdU. DAPI is excited by ultraviolet light (Du *et al*., 1998), and the blue emission is convenient for use as a counterstain in a single sample (Haugland, 1992).

### 2.10.7 Microscopy

After a final wash, sections were mounted onto chrome-alum-coated glass slides and allowed to air dry overnight before application of Vectashield® mounting medium (Vector Laboratories, Inc.) and coverslips.

Immunostaining was examined under an epi-fluorescent microscope (Zeiss Axio Imager. M1), and images captured by digital microphotography (Hamamatsu ORCA I-ER digital camera, Hamamatsu Photonics, Welwyn Garden City, Herts) and image analysis program (AxioVision, Zeiss Imaging Systems). The photographic panels in Chapters 6 and 7 were created using Microsoft PowerPoint 2007.

## 2.11 Statistical Analyses

Data collection, summary calculations, descriptive statistics, meal pattern calculations and syntax derivation were carried out using Microsoft Excel 2007. Grubb's Test was then used to detect outliers in the raw data. This test calculated the ratio Z as the difference between the outlier and the mean, divided by the standard deviation:

$$Z = \frac{\text{mean-value}}{SD}$$

Calculated Z values were then compared to a table of critical values (see Table 2.8) dependent on the total number of data points in a group (*n*). When the value of Z was higher than those tabulated the value of *p* was < 0.05 and the data point was excluded as an outlier.

**Table 2.8. Critical values for Z.**

| N | Critical Z | N | Critical Z |
|---|---|---|---|
| **3** | 1.15 | **27** | 2.86 |
| **4** | 1.48 | **28** | 2.88 |
| **5** | 1.71 | **29** | 2.89 |
| **6** | 1.89 | **30** | 2.91 |
| **7** | 2.02 | **31** | 2.92 |
| **8** | 2.13 | **32** | 2.94 |
| **9** | 2.21 | **33** | 2.95 |
| **10** | 2.29 | **34** | 2.97 |
| **11** | 2.34 | **35** | 2.98 |
| **12** | 2.41 | **36** | 2.99 |
| **13** | 2.46 | **37** | 3.00 |
| **14** | 2.51 | **38** | 3.01 |
| **15** | 2.55 | **39** | 3.03 |
| **16** | 2.59 | **40** | 3.04 |
| **17** | 2.62 | **50** | 3.13 |
| **18** | 2.65 | **60** | 3.20 |
| **19** | 2.68 | **70** | 3.26 |
| **20** | 2.71 | **80** | 3.31 |
| **21** | 2.73 | **90** | 3.35 |
| **22** | 2.76 | **100** | 3.38 |
| **23** | 2.78 | **110** | 3.42 |
| **24** | 2.80 | **120** | 3.44 |
| **25** | 2.82 | **130** | 3.47 |
| **26** | 2.84 | **140** | 3.49 |

Z is calculated using the Grubb's equation. The critical value of Z is then found in the table above, where N (*n*) is the number of values in the group. If Z is higher than the tabulated value, the *p* value is less than 0.05. Source: Barnett & Lewis, 1994. *Outliers in Statistical Data. Wiley Series in Probability & Statistics.* Wiley Publishing, Chichester, UK.

To determine position and dispersion (spread) of data, descriptive statistics (mean ± SEM) were first applied. All data within this thesis are presented as mean ± SEM unless otherwise stated.

Statistical analysis was carried out using software SPSS *v*19. It tests for equality of variance within groups (an assumption of parametric tests) and automatically carries out non-parametric tests (such as the Mann-Whitney U test), where this assumption fails. However, for the data presented in this thesis, it was unnecessary to use any non-parametric tests. Similarly, the data was automatically tested for normality.

Effects of diet treatment on several parameters including cumulative energy intake, cumulative body weight gain, percent change from baseline of plasma concentrations were determined by one-way ANOVA followed by *post hoc* Bonferroni correction (Chapters 3 and 5). Differences were considered significant at critical $p < 0.0166$, as is suitable for three comparisons (i.e. between control, high-SFA and high-PUFA diets). Additionally, area under the curve analysis, using the trapezoidal rule, was also carried out for satiety ratio.

Several measures were taken repeatedly over time from the same individual rats. These were food intake, body weight, plasma hormone and lipid concentrations and meal pattern parameters. Application of two-way ANOVA with repeated measures with post-hoc Bonferroni correction was applied to account for factors of time, diet and individual variation within animals. Results were considered significant at $p < 0.05$. A repeated measure is a term used when the same entities take part in all conditions of an experiment (Dytham *et al.,* 2003). For example here rats eat diets over several time points whereby certain measures are taken at each time point from each rat. In this type of experiment it is important to control for individual differences in tolerance to the diet: some rats can eat a lot of food without gaining weight and others will eat a small amount and gain weight. To control for these individual differences we can test the same rats in all conditions of the experiment so we test each rat after they have consumed the diet for one week, two weeks, or three weeks etc. Following each week's consumption several parameters (e.g. body weight, plasma hormones) are assessed from each rat monitoring the progression of the effects of the diet over time. Parametric tests based on the normal distribution

assume that data points are independent. This is not the case in a repeated measures design because data for different conditions have come from the same entities. This means that data from different experimental conditions will be related; because of this we have to make an additional assumption to those of the standard ANOVA. This assumption is known as the assumption of sphericity and assumes that the relationships between pairs of experimental conditions is similar (i.e. the level of dependence between pairs of groups is roughly equal). This process calculates the differences between results in all combinations of the treatment over time and then calculates the variance of these differences. Sphericity is met when these variances are roughly equal as is the case for all data presented here (Dytham *et al.,* 2003). If data do not meet this assumption then the degrees of freedom for the effect are reduced making the assessment more conservative. SPSS applies these adjustments automatically. In the case of a 2-way ANOVA with repeated measures; two independent variables are manipulated (diet type and time) and the same rats have been used across time to eat the three diet types. Therefore this analysis type is appropriate when you have two-repeated measures independent variables: each rat does both of the conditions in the experiment and provides results for each permutation of the two variables.

The effects of diet treatment on adipokine gene expression in white adipose tissue depots (Chapter 3) and muscle fibre-type ratios (Chapter 4) were determined by Student's 2-tailed *t*-test. Results were considered significant at $p < 0.05$.

## 2.12 Gantt Chart

As mentioned previously, work detailed in the following data chapters overlapped in places. A Gantt chart detailing this can be seen in Figure 2.21.

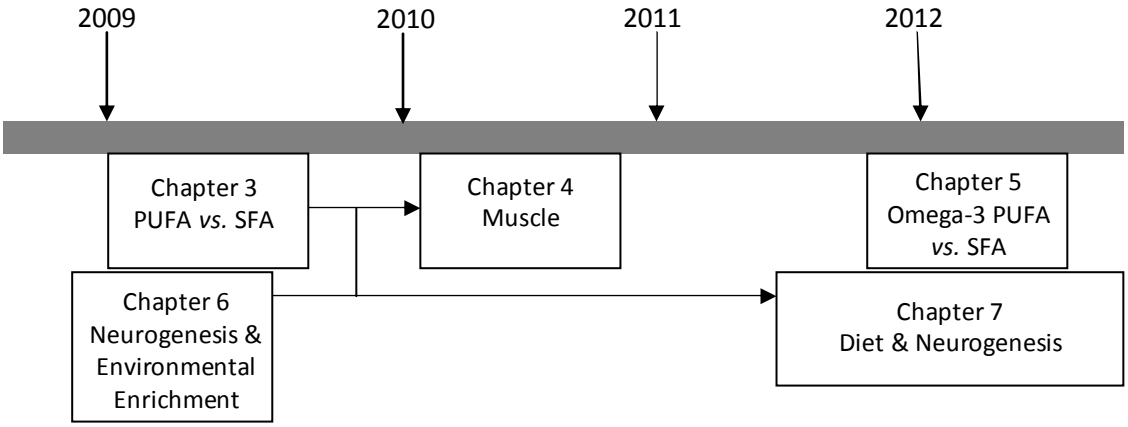



**Figure 2.21. Gantt chart of chronological ordering for studies detailed in Chapter 3-7.** Horizontal arrows indicate where protocols were applied, or tissues were analysed, between studies.

# CHAPTER 3

## CHARACTERISATION OF CHRONIC HIGH-PUFA FEEDING IN RAT

# Characterisation of Chronic High-PUFA Feeding in Rat

## 3.1 Introduction

Obesity is a risk factor for several metabolic disorders which impact on quality of life for an increasing number of people worldwide. Its phenotypic hallmark is elevated body weight, due to an abnormal level of adiposity (Schwartz & Brunzell, 1997; Seidell & Flegal, 1997). The development of obesity depends on the expression of a number of genes, largely determined by interaction with environmental factors. The nutrients we consume are among the most influential of these factors, and include fatty acids (FAs).

### 3.1.1 Effects of Dietary Fatty Acids on Adiposity

A diet high in fat will encourage weight gain, but the nature of this gain depends not only on the amount, but also the types of FAs consumed (Fernández-Quintela *et al.,* 2007). This appears to dictate not only the FA composition of the fat tissue, but also where fat is deposited in the body, which is of therapeutic interest because subcutaneous fat deposition is considered to be healthier than intra-abdominal (Field *et al*., 2008). Evidence from marine mammals, which consume a fish-based diet, suggests that polyunsaturated FAs (PUFAs) in fish oils accrue selectively in the subcutaneous depot (Brunborg *et al.,* 2006). Studies in rat have shown that consumption of diets consisting of elevated levels of fish oils containing the omega-3 PUFAs, EPA and DHA, protects against body fat gain (Belzung *et al.*, 1993; Hainault *et al.*, 1993; Hill *et al.*, 1993; Baillie *et al.*, 1999). This is important in terms of disease prevention, as, for example, the incidence of glucose intolerance, which precedes obesity-induced (type 2) diabetes in humans, is affected by both the amount and distribution of body fat (Kissebah *et al.*, 1982).

FAs exert their effects on adipose tissue at the level of the fat cell (adipocyte). They regulate gene transcription within the adipocyte to control the degree of body fat accumulation (Fernández-Quintela *et al.,* 2007): although findings in humans are inconsistent, rodent models have generally shown that "good" fats (PUFAs) can attenuate the accumulation of adipose tissue resulting from consumption of "bad" fats (saturated FAs, SFAs) (Buckley & Howe, 2009). They

do this by inhibiting expression of lipogenic genes and stimulating transcription of those involved in lipid oxidation (Fernández-Quintela *et al.,* 2007).

### 3.1.2 Adiposity Signals

Three key appetite-related hormones act to regulate body weight over the long-term, in part through control of body fat stores. Leptin, encoded by the *ob* gene, is one of a classic set of hormones (adipokines) secreted by the adipocyte, which inhibits appetite and regulates lipid metabolism to promote weight loss (Schwartz & Seeley, 1997). Another adipokine, adiponectin, helps to manage weight, blood sugar and cholesterol levels by acting in a feedback system to reduce appetite and prevent accumulation of excess blood sugar and fats (Hu *et al.*, 1996; Yang *et al.*, 2001; Chan *et al.*, 2005). In addition, the pancreatic hormone insulin increases uptake of lipids and glucose from the circulation into peripheral tissue, primarily adipose tissue and skeletal muscle, respectively (Ferrannini *et al.,* 2004; Fukuchi *et al.* 2004). Glucose is then used as an energy source and stops the body burning fat. Thus, insulin is an adipogenic hormone, such that high circulating levels are associated with fat deposition and weight gain (Pasquali & Vicemati, 2000; Catalano *et al.,* 2005). All three of these hormones can also influence brain sensitivity to satiety signals, such as gut hormones, as well as to nutrients *per* se; i.e. glucose and FAs (Woods & Lutz, 2006). However, the differential responses of these hormones in humans to individual dietary FAs remains unclear, as relatively few data from well-controlled studies are available. The few studies which have been conducted using the human population have been short-term, with small samples sizes, making it difficult to draw any valid conclusions (Buckley & Howe, 2009). On the other hand, experimental rodent models of obesity have successfully provided the means to study these relationships in controlled ways that, mainly for ethical and compliance reasons, are difficult in humans. However, studies to date vary considerably in a number of parameters, including species, duration and dietary fat content and type.

Dysregulated metabolism of these three key hormones results from excess SFA consumption and is characteristic of obesity resistance (Gray *et al*., 1993; Pasquali & Vicemati, 2000; Catalano *et al.,* 2005). Thus, measurement of their gene expression and circulating concentrations, while controlling for the sources of variation listed above, would make a fundamental contribution to the establishment

of valid animal models of FA feeding, and therefore, to our understanding of the potential mechanism by which dietary FA types differentially affect body weight metabolism.

### 3.1.3 Meal Patterns

Obesity is associated not only with numerous morphometric and metabolic abnormalities, such as those described above, but also behavioural ones. These include altered feeding behaviour, expressed as altered meal patterns (MPs) (Farshchi *et al.,* 2005). MPs can be described according to several measures, including length and frequency of meals, rate of feeding, total energy consumption, and satiety, the feeling of fullness experienced after a meal, which aids in the regulation of appetite and determines intermeal interval (IMI). These measures vary according to alterations in diet composition, including SFA content. For example, it has been shown that individuals consume greater amounts of energy from high-fat diets (HFDs) than from diets high in carbohydrates (Blundell *et al.,* 1994). Where this refers to high-SFA content specifically, as opposed to other FA types, this effect is known as passive overconsumption (Blundell & Burley, 1990) and is due to the high-energy density of high-fat foods (Blundell & Macdiarmid, 1997). There are a number of mechanisms behind this, one of which involves direct stimulation of appetite (Blundell & Burley, 1994). This has been demonstrated, for instance, by gavage feeding of oily SFAs in rats, which stimulates food intake and has been linked to the pleasant taste of the oil (Tordoff & Reed, 1991). Palatability overrides the acutely satiating effects of ingested fat (Lawton *et al*., 2000; Strubbs *et al.,* 2001), which result from the ability of fat-sensing in the gut to suppress ongoing feeding (Drewnowski, 1995). This is evidently a mechanism which eventually fails with chronic SFA consumption (Lawton *et al*., 2000; Strubbs *et al.,* 2001), explaining, at least in part, why SFAs exert a weaker effect on satiety than protein and carbohydrate (Blundell *et al.*, 1993; Blundell & Macdiarmid, 1997; Fernandez-Quintela *et al.*, 2007). Thus, in both humans and rodent models, obesity is associated with chronic consumption of diets high in SFAs and abnormal MPs, characterised by the intake of fewer, but larger, meals (Hariri & Thibault, 2011). In contrast, PUFAs, known to attenuate weight gain, have been revealed through human MP analysis to be more satiating than SFAs. However, this has been examined only under conditions where PUFAs have been consumed as a supplement, and only in the short

term (Lawton *et al*., 2000). In general, the metabolic effects of long-term consumption of PUFAs incorporated into foodstuffs need to be clarified, under controlled conditions employing isoenergetic, purified, and low-fat control diets. In particular, the MP signature of chronic PUFA intake has yet to be determined in either humans or animal models.

### 3.1.4 Beneficiaries

The successful treatment of obesity has been limited by the difficulty in developing acceptably safe and effective drug therapies (Field *et al.,* 2008). Natural interventions (nutraceuticals), such as the PUFAs found in fish oils, may be an alternative approach which encourages compliance in human patients. If their associated health claims could be substantiated by proven molecular and physiological changes favouring healthy body composition and enhanced satiety, the food industry would be encouraged to further develop methods for incorporation into widely consumed foodstuffs.

### 3.1.5 Research Question

***Does chronic consumption of a diet highly enriched in PUFAs improve energy homeostasis in rat?***

### 3.1.6 Predictions

If so, then significant differences would be observed between morphometric, metabolic and behavioural indices of energy metabolism in rats fed high-PUFA diets long-term, compared to those fed high-SFA or low-fat, control diets.

### 3.1.7 Aims

To test these predictions by assessing diet-induced differences in

1. body weight and composition associated with
2. corresponding alterations in energy intake and meal patterns and
3. circulating concentrations of appetite-related factors.

### 3.1.8 Expected Outcomes

PUFA-fed rats would display

1. attenuated weight gain,
2. improved fat-to-lean ratio,
3. reduced energy intake,
4. changes in meal patterns indicating enhanced satiety, and
5. reduced circulating concentrations of triglycerides, leptin and insulin, and increased concentrations of adiponectin.

It was important to bear in mind that these expectations would have to be balanced against the potential effects of the increased energy content of the chosen high-PUFA diet compared to that of the low-fat, control diet, which might occur independently of, and possibly override, the effects of the specific PUFAs present in the diet (Hill *et al.,* 1993).

## 3.2 Materials and Methods

### 3.2.1 Animals and Dietary Treatment

Age-matched adult male Wistar rats (~250 g) were stratified by body weight and randomly assigned to one of two groups (*n*=8/group, except where otherwise indicated; see meal pattern data acquisition below). For eight weeks, they were fed isoenergetic diets, enriched with either SFAs or PUFAs. Both diets provided 40% of energy from fat, mainly from lard and fish oil, respectively. These acted as controls for one another by eliminating the potentially confounding factor of differing energy intake. A third group of adult male Wistar rats (~250 g; also *n*=8), fed standard (low-fat) chow (providing 10% of energy from fat, from soybean oil) was run separately several months later, when its need was realised and funds became available. It acted as a control for obesity induction, demonstrating normal weight gain and food intake. All diets were manufactured by Research Diets, Inc.™ (NJ, USA). Body weight was measured weekly and food and water intake daily. Details of general husbandry and maintenance can be found in Chapter 2, Section 2.1.6. A summary of the diet compositions can be seen in Table 3.1. Full nutritional breakdowns are given in Appendix I & II.

**Table 3.1. Nutritional composition of low-fat (control) and isoenergetic high-fat diets.**

| | Diet Type | | |
|---|---|---|---|
| **Macronutrient content** | **Control** | **SFA-enriched** | **PUFA-enriched** |
| Total energy content (kcal/gm) | 3.85 | 4.58 | 4.58 |
| % energy from carbohydrates | 70 | 40 | 40 |
| % energy from protein | 20 | 20 | 20 |
| % energy from fat | 10 | 40 | 40 |
| % energy from fat as SFAs | 23 | 36 | 27 |
| % energy from fat as PUFAs | 48 | 19 | 52 |
| % energy from MUFAs | 29 | 45 | 21 |
| % energy from fat as EPA & DHA (omega-3) | 0 | 0 | 25 |
| % energy from sucrose | 10 | 10 | 10 |

The high-PUFA diet and its high-fat control, enriched in SFAs, contained equal amounts of energy overall (isoenergetic) and contributed equal amounts of energy from fat. The fat content in both diets was a mixture of PUFAs and SFAs. Only the high-PUFA diet contained omega-3 fatty acids. **Abbreviations:** DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

### 3.2.2 BrdU Administration

Rats received the cell proliferation tracer, bromodeoxyuridine (BrdU) dissolved in their drinking water (1 mg/ml) for the last seven days of the study, at which time a single play tube was introduced to the cage as an enrichment tool. The results of this part of the study are presented and discussed in Chapter 7.

### 3.2.3 Meal Pattern Data Acquisition and Analysis

Meal pattern information was gathered from rats ($n$=4/group) every two weeks over three-day periods for the duration of the study. Group size was limited by available cage numbers and logistical constraints of the study. Rats were removed from home cages and placed in specialised cages equipped with automated food intake monitoring systems, where they were given access to diets and water *ad libitum*. Raw data acquired in diurnal and nocturnal phases were analysed and are displayed according to the following previously published parameters: total energy intake (kJ), number and duration (min) of feeding episodes (hereafter referred to as 'meals'), rate of feeding (g/min), intermeal interval (IMI; min) and satiety ratio (IMI/average

energy content of the previous meal consumed; min/kJ) (Farley *et al*., 2003; Cattone *et al.*, 2007; Hariri & Thibault, 2011). Details of the cage and software analysis system can be found in Chapter 2, Section 2.4.3.

### 3.2.4 Blood Chemistry

After an overnight fast, and under brief gaseous anaesthesia, blood samples (200µl) were collected from the tail vein at baseline and fortnightly thereafter and plasma separated by centrifugation. Blood glucose concentrations were measured immediately by glucose-oxidase strips and a hand-held glucose meter. Plasma concentrations of triglycerides (TGs) were measured by diagnostic (enzymatic, colorimetric) kit and concentrations of leptin, adiponectin and insulin by ELISA, according to manufacturers' protocols, except where adapted to in-house specifications. Details of suppliers and manufacturers' protocols can be found in Chapter 2, section 2.6).

### 3.2.5 Termination & Body Composition Analysis

Rats were perfuse-fixed and brains removed for histological analysis of BrdU uptake and other cell proliferation markers, as described in Chapter 2 (Section 2.9), where details of anaesthetic protocols can also be found (Section 2.2.1). Results of these analyses are presented and discussed in Chapter 7. The descending aorta was clamped to prevent fixation of peripheral tissues required fresh. White adipose tissue (WAT) pads, interscapular brown adipose tissue (BAT), gastrocnemius and soleus muscle and liver were dissected free, weighed, snap-frozen in liquid nitrogen, and stored at -80°C for further analysis. Tissue weights were expressed as a percentage of final body weight.

### 3.2.6 Real-Time PCR

WAT samples from PUFA- and SFA-fed rats were homogenised and RNA extracted using the TRIReagent® method. RNA was then reverse-transcribed on a thermal cycler with reverse transcriptase enzyme to create cDNA. All PCR reactions were performed using a real-time PCR machine and data collected and analysed automatically by the integral software at the end of each run. Gene expression was determined by relative quantification with the 2-ΔΔCT method (Livak & Schmittgen, 2001). Extraction and reactions were carried out according to

manufacturers' protocols. Details of reaction stages, equipment and suppliers can be found in Chapter 2, location 2.8. The low-fat control group was run separately and adipose samples collected and stored for future analysis.

### 3.2.7 Statistical Analysis

Data are expressed as mean ± SEM. All tissue masses are presented as a relative percentage of final body weight. Differences between diet groups in three-way comparisons were determined using one-way ANOVA with post-hoc Bonferroni correction and considered significant at critical $p<0.0166$ for 3 comparisons. Differences between diet groups over a time course were determined using 2-way ANOVA with repeated measures and post-hoc Bonferroni correction and considered significant at $p<0.05$. In all instances comparisons demonstrated roughly equal variation and therefore the sphericity criteria were met and consequently variation data is not presented here. Differences in two-way comparisons were determined by Student's 2-tailed *t*-test, and considered significant at $p<0.05$. Statistical analysis was carried out using software SPSS *v*19. Between-group comparisons of satiety time course data were made after first quantifying by calculation of area under the curve (AUC; StatsDirect software), using the trapezoidal rule. Justification for use of all statistical analyses can be found in Chapter 2 section 2.11.

## 3.3 Results

### 3.3.1 Energy Intake & Body Weight

PUFA- and SFA-fed rats consumed comparable amounts of energy overall ($p>0.05$), which were greater than that consumed by controls (+27±2.1% for SFA-fed and +29±0.8% for PUFA-fed, $p<0.01$; Figs. 3.1A & B). Daily energy intake remained constant in control animals throughout the study, whereas that in both high-fat-fed groups reduced progressively, after a transient initial increase in the first week of feeding (Fig. 3.1A). This would appear to account for their greater cumulative intake, as there were no differences in absolute body weight or weight gain compared to controls (Figs. 3.2A & B). Indeed, weekly body weight gain was reduced comparably in all diet groups as the study progressed (Fig. 3.2C). Together, these outcomes were reflected in equally reduced food efficiency in both high-fat-fed groups (-28±1.2% and –24±1.7% *vs.* controls in SFA and PUFA groups, respectively; both $p<0.01$; Fig. 3.2D)

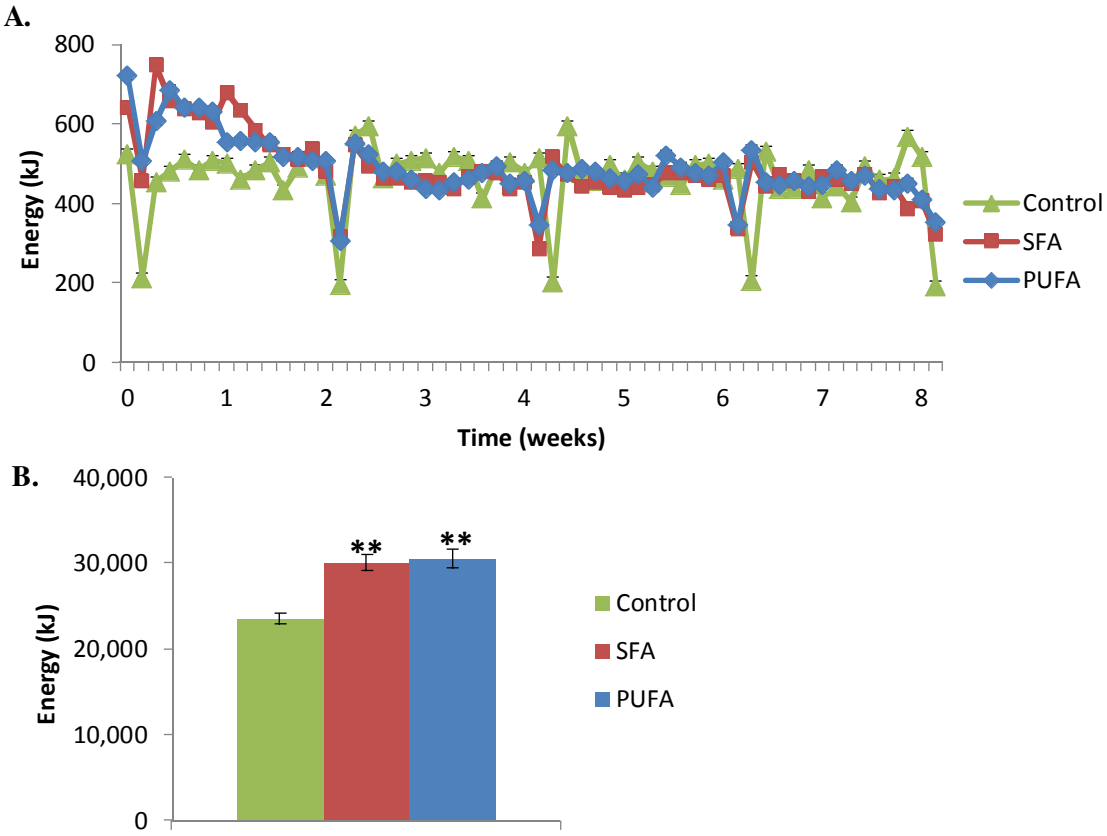



**Figure 3.1.** Energy intake evolution (A) and cumulative energy intake (B) in control, SFA- and PUFA-fed rats consuming diets for eight weeks. Reduced intake at two-week intervals (A) reflects imposed overnight fasts prior to blood sampling. **Overall energy intake was increased equally in the high-fat-fed groups.** Values are expressed as mean ± SEM ($n$=8/group); **$p<0.01$ compared to controls (1-way ANOVA).

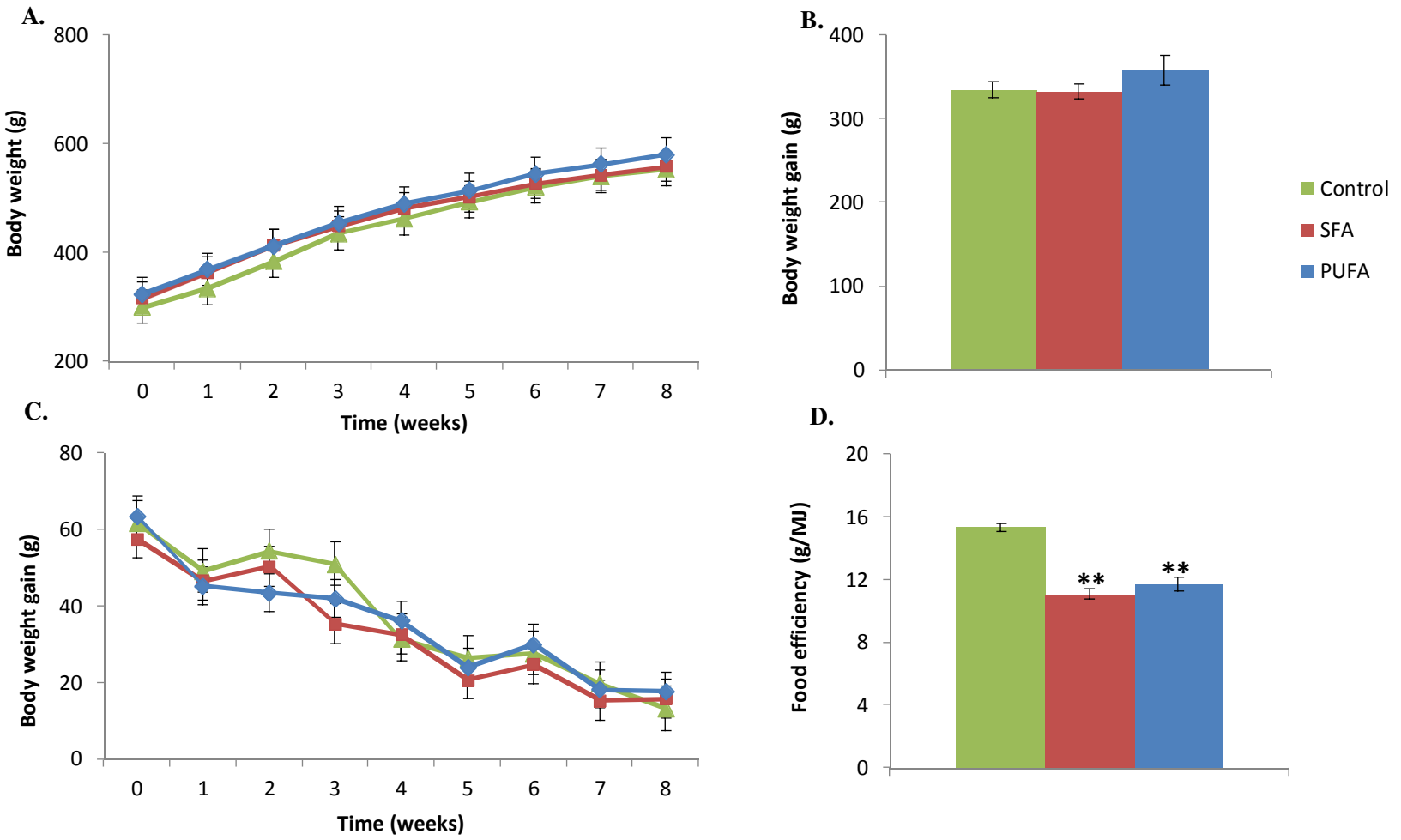



**Figure 3.2. Absolute** body weight evolution (A), cumulative weight gain (B), weight gain evolution (C) and food efficiency (D) in control, SFA- and PUFA-fed rats consuming diets for eight weeks. **Diet had no effect on body weight, but food efficiency was reduced equally in the high-fat-fed groups.** Values are expressed as mean ± SEM ($n$=8/group); **$p$<0.01 compared to controls (1-way ANOVA). Food efficiency = Δbody weight (g)/Σfood intake (MJ).

### 3.3.2 Terminal Tissue Mass and Body Composition

Despite the lack of effect on body weight, adiposity was increased in both high-fat-fed groups. Comparable increases in WAT accrual were observed between the two groups in the perirenal depot (+60±1.9% for SFA-fed and +50±2.5% for PUFA-fed rats *vs.* controls; both $p<0.01$; Fig. 3.3A) and inguinal depots (+59±3.1% for SFA-fed and +68±2.9% for PUFA-fed *vs.* controls; both $p<0.01$; Fig. 3.3A). The epididymal depot was increased in SFA-fed animals only (+46±4.1% *vs.* controls; $p<0.01$; Fig. 3.3A). BAT mass was unchanged, as were skeletal muscle masses (Figs. 3.3B, D). Body composition was altered accordingly by both types of high-fat feeding, which increased the ratio of fat-to-lean tissue (+91% and +70%, SFA and PUFA *vs.* controls, respectively; Fig. 3.3E). Liver mass was increased in PUFA-fed rats compared to both controls and SFA-fed rats (+33% and +16%, respectively; both $p<0.01$; Fig. 3.3C) and paler in colour, suggesting an increase in fat deposition.

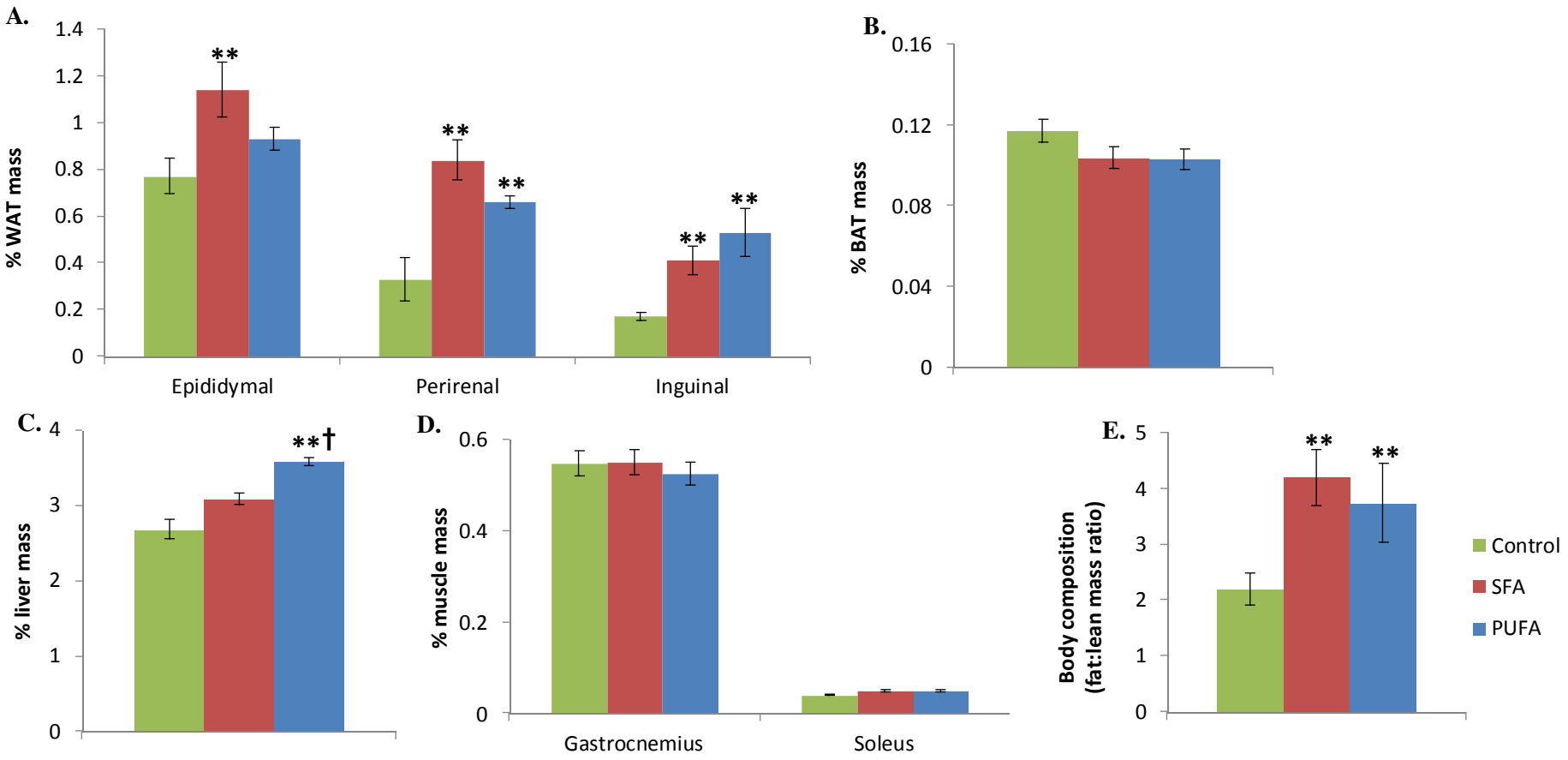



**Figure 3.3.** Tissue mass relative to final body weight in control, SFA- and PUFA-fed rats after consumption of diets for eight weeks: WAT (A), BAT (B), liver (C) and skeletal muscle (D). Body composition (E) was calculated as the ratio of summed WAT depot to muscle masses. Both types of high-fat feeding led to **accumulation of WAT in all depots, reflected in altered body composition, and increased liver mass.** Values are displayed as mean ± SEM ($n$=8/group); **$p$<0.01 compared to controls; †$p$<0.01 compared to SFA (1-way ANOVA).

### 3.3.3 Circulating Factors

#### 3.3.3.1 Leptin - Plasma Concentrations and Gene Expression

Analysis by two-way ANOVA with repeated measures demonstrated a significant difference in circulating leptin concentrations between diet groups ($F(2, 21) = 32.33$, $p<0.0001$), over time ($F(4, 84) = 27.04$, $p<0.0001$) and a significant interaction between diet and time ($F(8, 84) = 3.968$, $p=0.0005$). Consistent with increased adiposity, both high-fat-fed groups had become comparably hyperleptinaemic by two weeks of consumption, demonstrating an increase from baseline in circulating concentrations of leptin of 160±8.9% in SFA-fed rats and 157±5.2% in PUFA-fed rats. This was sustained throughout the remainder of the study, such that at termination, both high-fat-fed groups showed a 63±2.4% increase above control concentrations (both $p<0.05$; Fig. 3.4).

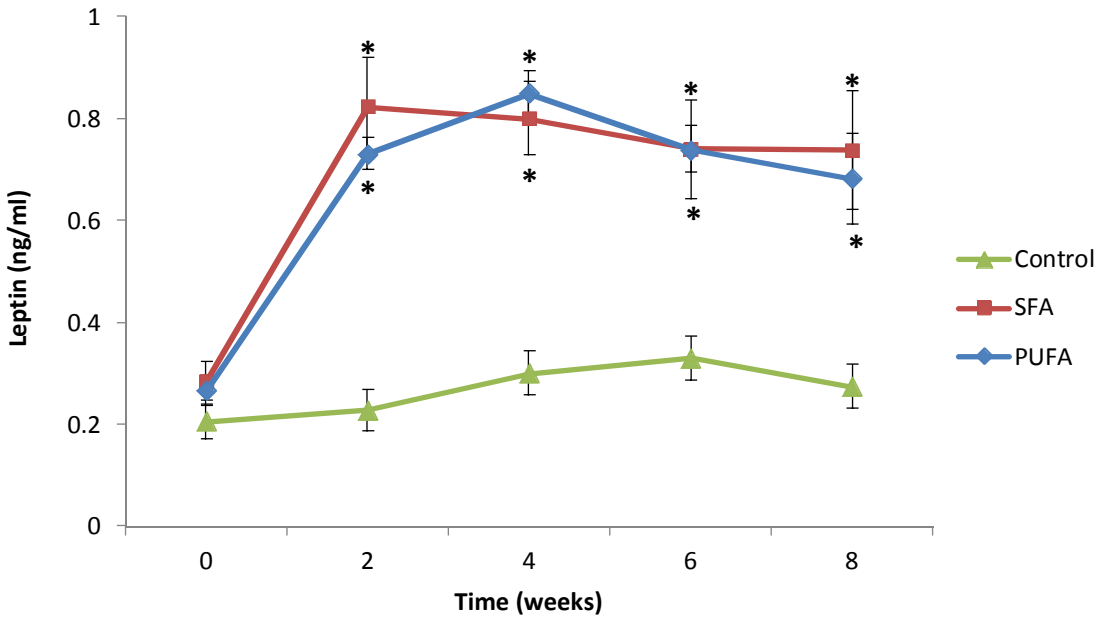



**Figure 3.4.** Plasma leptin concentrations in control, SFA- and PUFA-fed rats consuming diets for eight weeks. **High-fat-fed groups were equally hyperleptinaemic by the second week of consumption.** Values are expressed as mean ± SEM ($n$=8/group); *$p<0.05$ compared to controls (2-way ANOVA with repeated measures).

In contrast to equal circulating levels of leptin, gene expression was reduced in PUFA- compared to SFA-fed rats in all adipose depots examined. Although this difference was significant in the epididymal and inguinal depots (Fig. 3.5), all mean values were less than one relative fold change and therefore, not considered to reflect a biologically significant difference (Glick & Pasternak, 2003; Logan *et al.,* 2009).

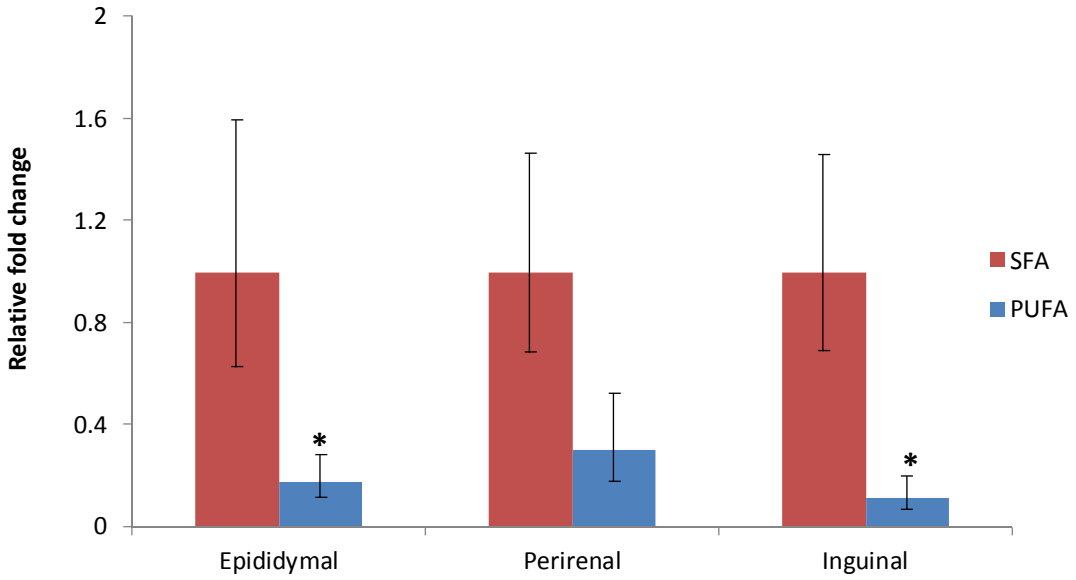



**Figure 3.5.** Terminal WAT leptin gene expression in SFA- and PUFA-fed rats consuming diets for eight weeks. **Differential effects on leptin expression were observed in all fat depots.** However, although only those reductions by high-PUFA feeding in the epididymal and inguinal depots were statistically significant, they were not considered biologically meaningful (see text). All values are expressed as mean ± SEM ($n$=8/group); *$p$<0.05 compared to SFA-fed controls (Student's 2-tailed $t$-test). *In this instance SFA-fed rats act as a control for PUFA-fed rats.*

### 3.3.3.2 Adiponectin - Plasma Concentrations and Gene Expression

Analysis by two-way ANOVA with repeated measures demonstrated no differences in circulating adiponectin concentrations between diet groups ($F(2, 21) = 1.811$, $p=0.1881$), over time ($F(4, 84) = 1.772$, $p=0.1420$) or any interaction between diet and time ($F(8, 84) = 0.7459$, $p=0.6509$). Circulating plasma adiponectin concentrations were similar for all dietary groups at all time points for the duration of the study (all $p>0.05$). By the end of the study there was a slight increase in concentrations from baseline for all three dietary groups (+26±2.1% for controls, +19±4.7% for SFA-fed rats and +27±1.8% for PUFA-fed rats; see Fig. 3.6).

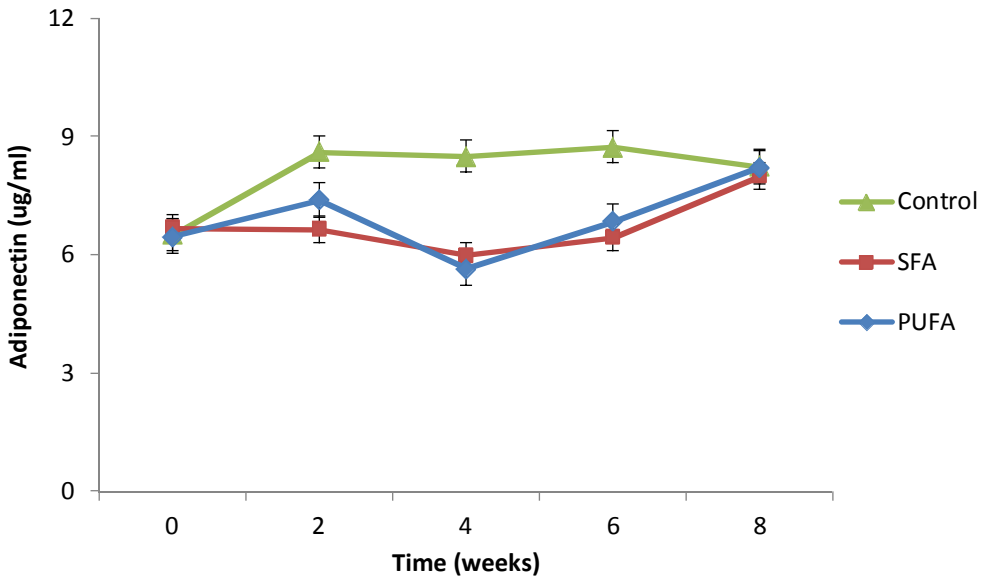



**Figure 3.6.** Plasma adiponectin concentrations in control, SFA- and PUFA-fed rats consuming diets for eight weeks. **High-fat feeding had no effect.** Values are expressed as mean ± SEM ($n=8$/group); all $p>0.05$ (2-way ANOVA with repeated measures).

Consistent with the comparable circulating levels of adiponectin, gene expression was also similar in epididymal, perirenal and inguinal adipose depots of PUFA-fed animals compared to those of SFA-fed animals (all $p>0.05$; Fig. 3.7).

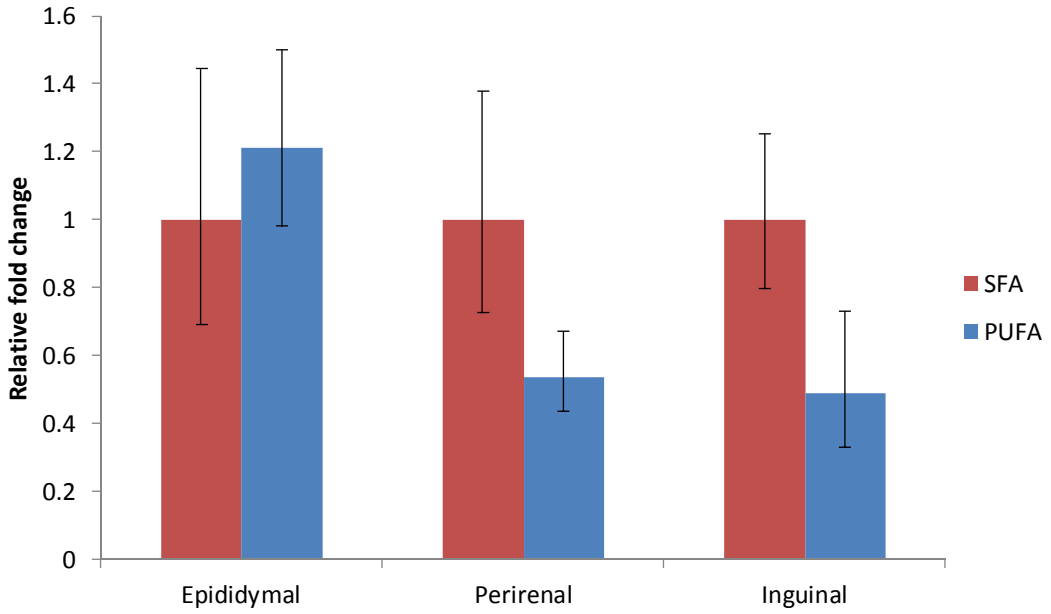



**Figure 3.7.** Terminal WAT adiponectin gene expression in SFA- and PUFA-fed rats consuming diets for eight weeks. **High-fat feeding did not differentially affect expression.** All values are expressed as mean ± SEM ($n$=8/group); all $p>0.05$ (Student's 2-tailed $t$-test). *In this instance SFA-fed rats act as a control for PUFA-fed rats.*

### 3.3.3.3 Whole-Blood Glucose Concentrations

Analysis by two-way ANOVA with repeated measures demonstrated no differences in whole-blood glucose concentrations between diet groups ($F(2, 21) = 3.587$, $p=0.0921$), over time ($F(4, 84) = 2.287$, $p=0.0667$) or any interaction between diet and time ($F(8, 84) = 3.423$, $p=0.0911$). There were no differences in whole-blood glucose concentrations between diet groups over the course of the study (all $p>0.05$ *vs.* controls). In addition, within-group concentrations at termination had changed very little from baseline: +6±0.4%, -16±1.1% and -14±1.4% in control, SFA- and PUFA-fed groups, respectively (Fig. 3.8).

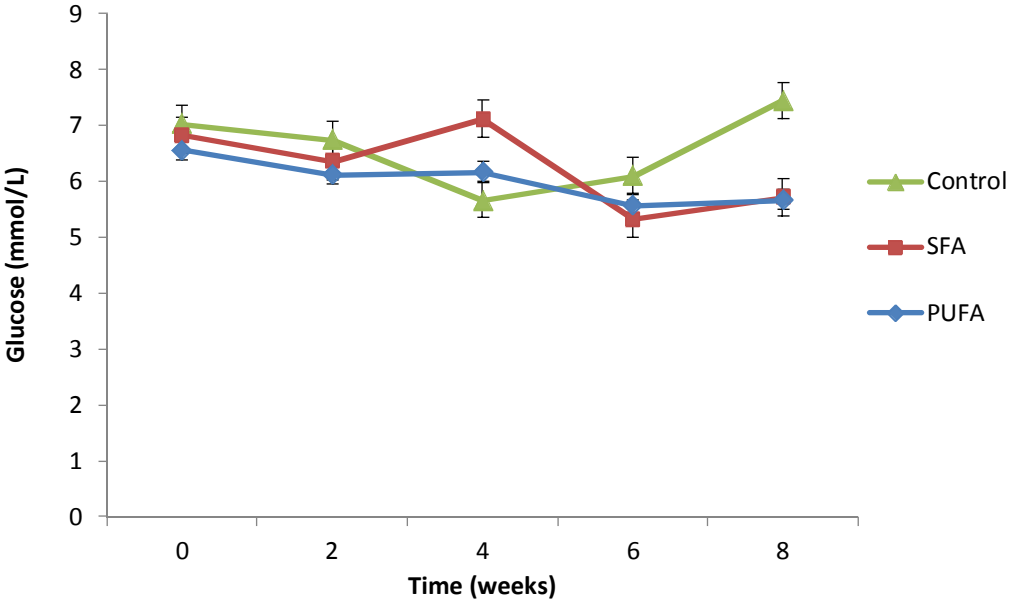



**Figure 3.8.** Fasting whole-blood glucose concentrations in control, SFA- and PUFA-fed rats consuming diets for eight weeks. **High-fat feeding had no effect.** Values are expressed as mean ± SEM ($n=8$/group); all $p>0.01$ (2-way ANOVA with repeated measures).

### 3.3.3.4 Plasma Insulin Concentrations

Analysis by two-way ANOVA with repeated measures demonstrated a significant difference in circulating insulin concentrations between diet groups ($F(2, 21) = 66.50$, $p<0.0001$), over time ($F(4, 84) = 318.1$, $p<0.0001$) and a significant interaction between diet and time ($F(8, 84) = 17.56$, $p<0.0001$). All groups became progressively hyperinsulinaemic, with terminal plasma insulin concentrations having increased from baseline by 260±8.5% in controls, 520±6.2% in SFA-fed animals and 432±7.4% in PUFA-fed animals. Concentrations were significantly raised above those of controls by four weeks' exposure to the high-fat diets (+118±8.4% in SFA-fed and +91±6.7% in PUFA-fed rats *vs.* controls) and remained so until termination at week eight (both $p<0.05$; Fig. 3.9).

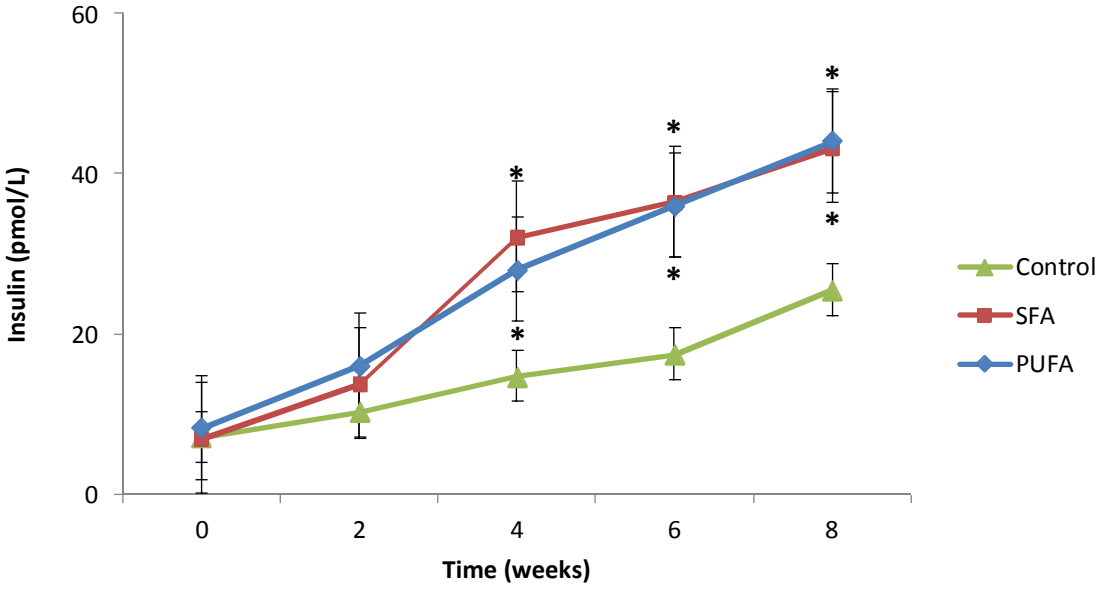



**Figure 3.9.** Plasma insulin concentrations in control, SFA- and PUFA-fed rats consuming diets for eight weeks. **All groups became progressively hyperinsulinaemic. Both high-fat diets induced equivalent hyperinsulinaemia, exceeding that of controls by six weeks of consumption.** Values are expressed as mean ± SEM ($n$=8/group); *$p<0.05$ compared to controls (2-way ANOVA with repeated measures).

### 3.3.3.5 Plasma Triglyceride Concentrations

Analysis by two-way ANOVA with repeated measures demonstrated a significant difference in circulating triglyceride concentrations between diet groups ($F(2, 21) = 17.56$, $p<0.0001$) and over time ($F(4, 84) = 7.462$, $p<0.0001$), but no interaction between diet and time ($F(8, 84) = 1.933$, $p<0.0655$). Despite the strong association between high SFA intake and increased circulating triglycerides (e.g. Ghibaudi, *et al.* 2002), concentrations remained consistent throughout. In contrast, PUFA-fed animals exhibited significantly reduced concentrations in comparison to both control rats, and unlike other circulating factors examined, to SFA-fed animals as well (-44±0.8% and –51±1.2%, respectively, at termination; both $p<0.05$; Fig. 3.10). The reduction in triglycerides for PUFA-fed rats was apparent by two weeks of consumption and was sustained throughout the study (-44±3.9% from baseline).

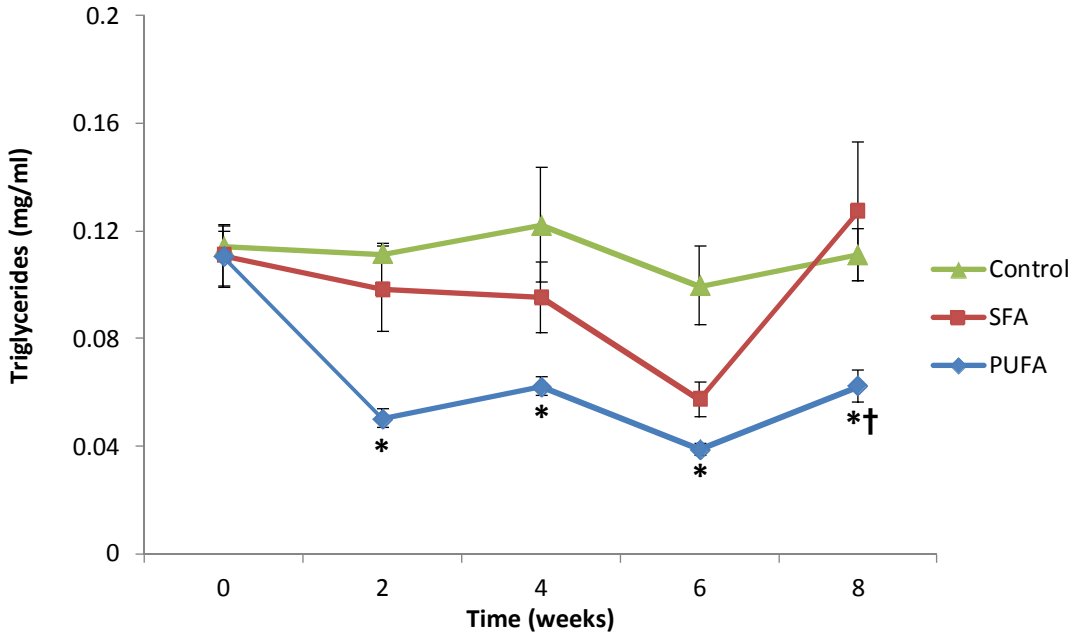



**Figure 3.10.** Plasma triglyceride concentrations in control, SFA- and PUFA-fed rats consuming diets for eight weeks. **High-PUFA-feeding reduced circulating triglyceride concentrations throughout dietary exposure.** Values are expressed as mean ± SEM ($n$=8/group); *$p<0.05$ compared to controls; †$p<0.05$ compared to SFA (2-way ANOVA with repeated measures).

### 3.3.4 Meal Patterns

High-fat feeding appeared to have no effect on diurnal or nocturnal energy intake when analysed separately over the duration of the study (Fig. 3.11A), in contrast to the increase observed in cumulative 24-hour intake (Fig. 3.1B). As expected in a nocturnal species, diurnal intake was consistently less than nocturnal intake (-78±2.1% for SFA-fed rats and -79±1.6% for PUFA-fed rats, $p<0.05$; Fig. 3.11A), reflected in greater intermeal interval (IMI; Figs. 3.12A, B). As the study progressed, nocturnal energy intake and number of meals gradually decreased. (Figs. 3.11A, B). Similarly, there were no differences between diet groups in meal duration or feeding rate (Figs. 3.11C, D), though there was an increase in rate over time from baseline for all groups (nocturnal, +41±0.9% for SFA-fed rats and +40±1.2% for PUFA-fed rats; diurnal, +93±2.1% for SFA-fed rats and +93±1.9% for PUFA-fed rats).

In addition, although high-fat feeding had no overall effect on IMI in either phase (AUC; all $p>0.01$; Figs. 3.12A, B), it induced an acute (non-significant) increase in diurnal IMI in the early part of the study, between weeks 1 and 3, which did not persist. Diurnal IMI in control animals also increased and then approached baseline toward the end of the study, but gradually (Fig. 3.12A). IMI changes over time in both phases were mirrored by changes in satiety (Figs. 3.12C, D). The enhancement in diurnal IMI and satiety observed between weeks 1 and 3 corresponds to the decline in energy intake observed over the same period (Fig. 3.1A). Again reflecting the natural activity patterns of rodents, and consistent with reduced diurnal energy intake and number of meals (Figs. 3.11A, B), IMI and satiety were greater in the diurnal than the nocturnal phase, irrespective of diet (e.g. PUFA-fed satiety ratio AUC; +98±0.6%; $p<0.01$). Satiety was markedly enhanced, by more than two-fold, throughout the diurnal phase in PUFA-fed, compared to control and SFA-fed, animals (AUC; +217±4.8% *vs.* controls, $p<0.01$; Fig. 3.12E).

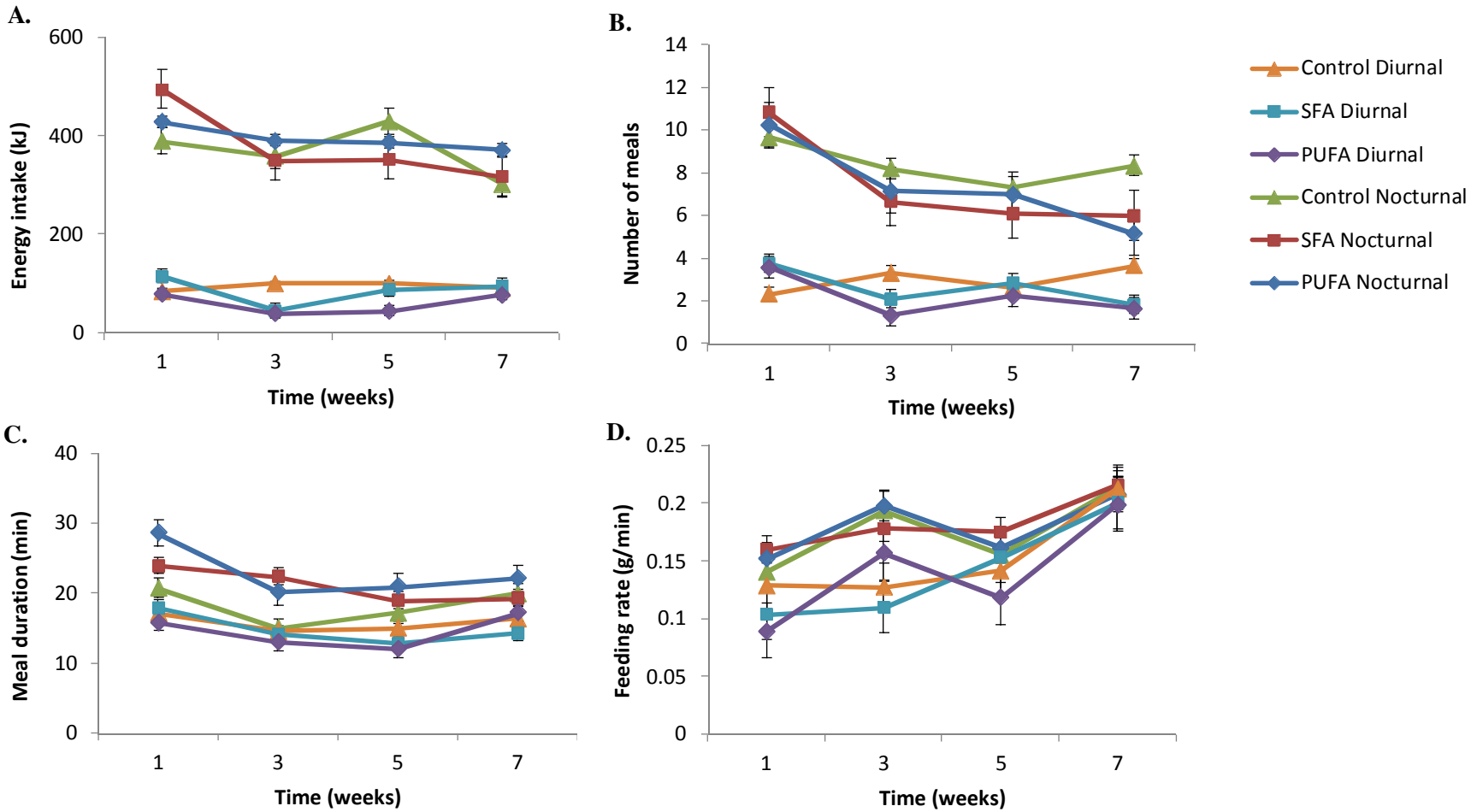



**Figure 3.11.** Diurnal and nocturnal energy intake (A), number of meals (B), meal duration (C) and feeding rate (D) in control, SFA- and PUFA-fed rats consuming diets for eight weeks. **Diet had no effect on these meal pattern parameters.** Values are expressed as mean ± SEM ($n$=4/group); all $p$>0.05 (2-way ANOVA with repeated measures).

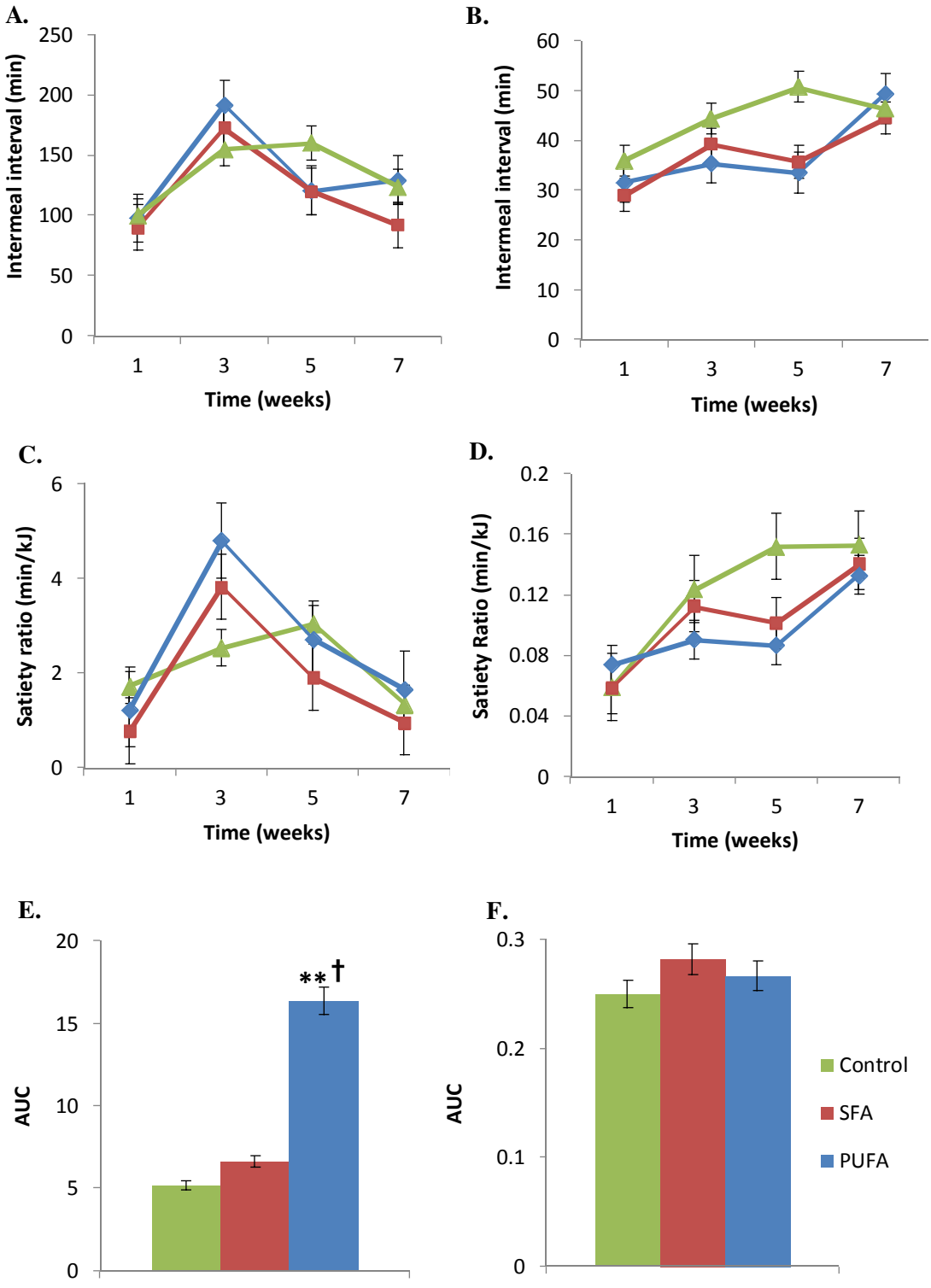



**Figure 3.12.** Diurnal and nocturnal intermeal interval (A, B) and satiety ratio (C, D) in control, SFA- and PUFA-fed rats consuming diets for eight weeks. Summary measure of satiety over time is expressed as area under the curve (AUC; E, F). **High-PUFA feeding enhanced diurnal satiety.** Values are expressed as mean ± SEM ($n$=4/group); **$p$<0.01 compared to controls; †$p$<0.01 compared to SFA (1-way ANOVA).

### 3.3.5 Circadian Rhythmicity

The patterns of feeding rhythmicity stimulated by each diet across the study period are displayed in Fig. 3.13. They clearly support the summary data indicating a gradual reduction in energy intake and number of meals in all diet groups over the course of the study (Figs. 3.13A-D *vs.* Figs. 3.11A, B), and in addition, reveal subtle alterations over the study weeks in distribution of meals, particularly across the nocturnal phase. These shifts can be more fully appreciated by examining ranges of values associated with related parameters of feeding activity (Table 3.2) and become particularly clear when qualitatively comparing activity between the first (week 1) and final weeks (week 7) of analysis (Fig. 3.13A *vs.* D).

The most noteworthy changes were the disappearance after week 1 of the large meal taken by control animals just prior to the onset of the nocturnal phase ('lights off'; Fig. 3.13A *vs.* B-D). Diurnal intake did not persist in any group, but fell below the level of detection, as defined by automated cage software criteria, sooner in high-fat-fed animals than controls (after week 1 *vs.* week 3; Figs. 3.13A *vs.* B). Secondly, all groups began to consume meals within a more restricted nocturnal window after week 1 (Fig. 3.13A), which became most pronounced at week 5 in the high-fat-fed groups (Fig. 3.13C), before expanding slightly again in the final week 7. In contrast, this clustering became most pronounced later in controls, at week 7 (Fig. 3.13D). This narrower window of activity occurred in the middle hours of the night, with the disappearance of meals initially observed in week 1 from the periods just after 'lights off' and just before 'lights on' the next day. Aside from the widest range of meal sizes being consumed by the high-SFA-fed group (Table 3.2), there did not appear to be any marked differences in feeding rhythmicity between the two high-fat groups.

**Table 3.2. Ranges of values associated with the circadian rhythmicity of feeding.**

| *Diurnal* | *Week 1* | | | *Week3* | | | *Week 5* | | | *Week 7* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Range** | **Control** | **SFA** | **PUFA** | **Control** | **SFA** | **PUFA** | **Control** | **SFA** | **PUFA** | **Control** | **SFA** | **PUFA** |
| **Consumption window** | 15.30-17.30 (4 hours) | 14.30-15.00 (0.5 hours) | 17.00-17.30 (0.5 hours) | 18.30-19.30 (1 hour) | - | - | - | - | - | - | - | - |
| **Number of meals** | 2 | 1 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Energy intake/range of meal (kJ)** | 11-49kJ (38kJ) | 6kJ | 5kJ | 6-19kJ (12.5kJ) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Nocturnal* | *Week 1* | | | *Week 3* | | | *Week 5* | | | *Week 7* | | |
| **Range** | **Control** | **SFA** | **PUFA** | **Control** | **SFA** | **PUFA** | **Control** | **SFA** | **PUFA** | **Control** | **SFA** | **PUFA** |
| **Consumption window** | 20.30-05.30 (9 hours) | 20.00-07.00 (11 hours) | 20.30-06.00 (9.5 hours) | 20.00-06.30 (9 hours) | 20.30-07.30 (11 hours) | 21.00-07.00 (10 hours) | 20.00-05.00 (10 hours) | 23.30-06.00 (6.5 hours) | 22.30-06.00 (7.5 hours) | 22.30-05.00 (7.5 hours) | 21.30-05.30 (9 hours) | 21.00-06.00 (9 hours) |
| **Number of meals** | 9 | 11 | 12 | 11 | 13 | 15 | 9 | 10 | 11 | 10 | 8 | 9 |
| **Energy intake/range of meal (kJ)** | 20-50kJ (30kJ) | 19-54kJ (35kJ) | 15-54kJ (39kJ) | 18-36kJ (18kJ) | 23-43kJ (20kJ) | 12-42kJ (30kJ) | 20-36kJ (16kJ) | 20-38kJ (18kJ) | 5-41kJ (36kJ) | 15-31kJ (16kJ) | 17-26kJ (9kJ) | 11-34kJ (23kJ) |

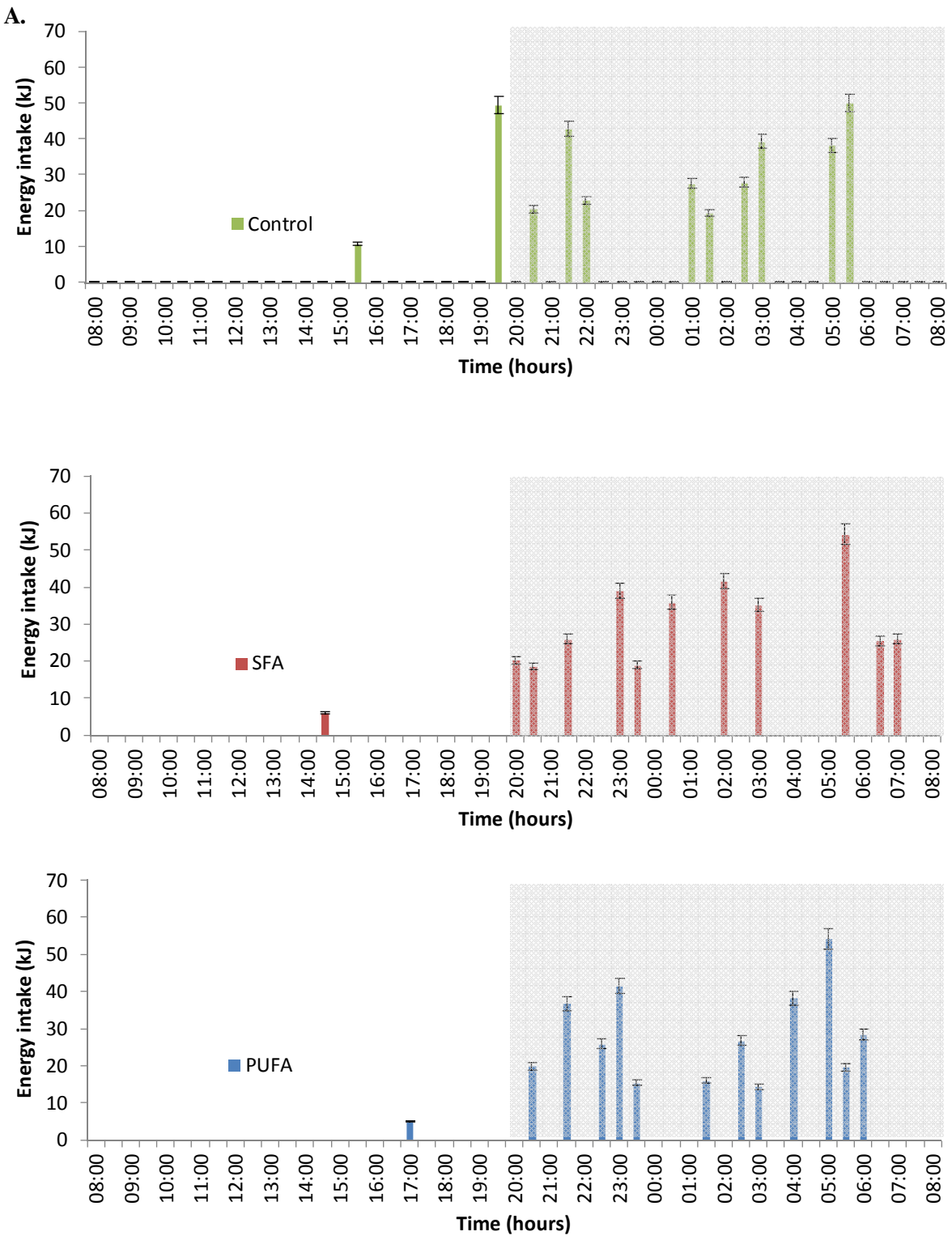
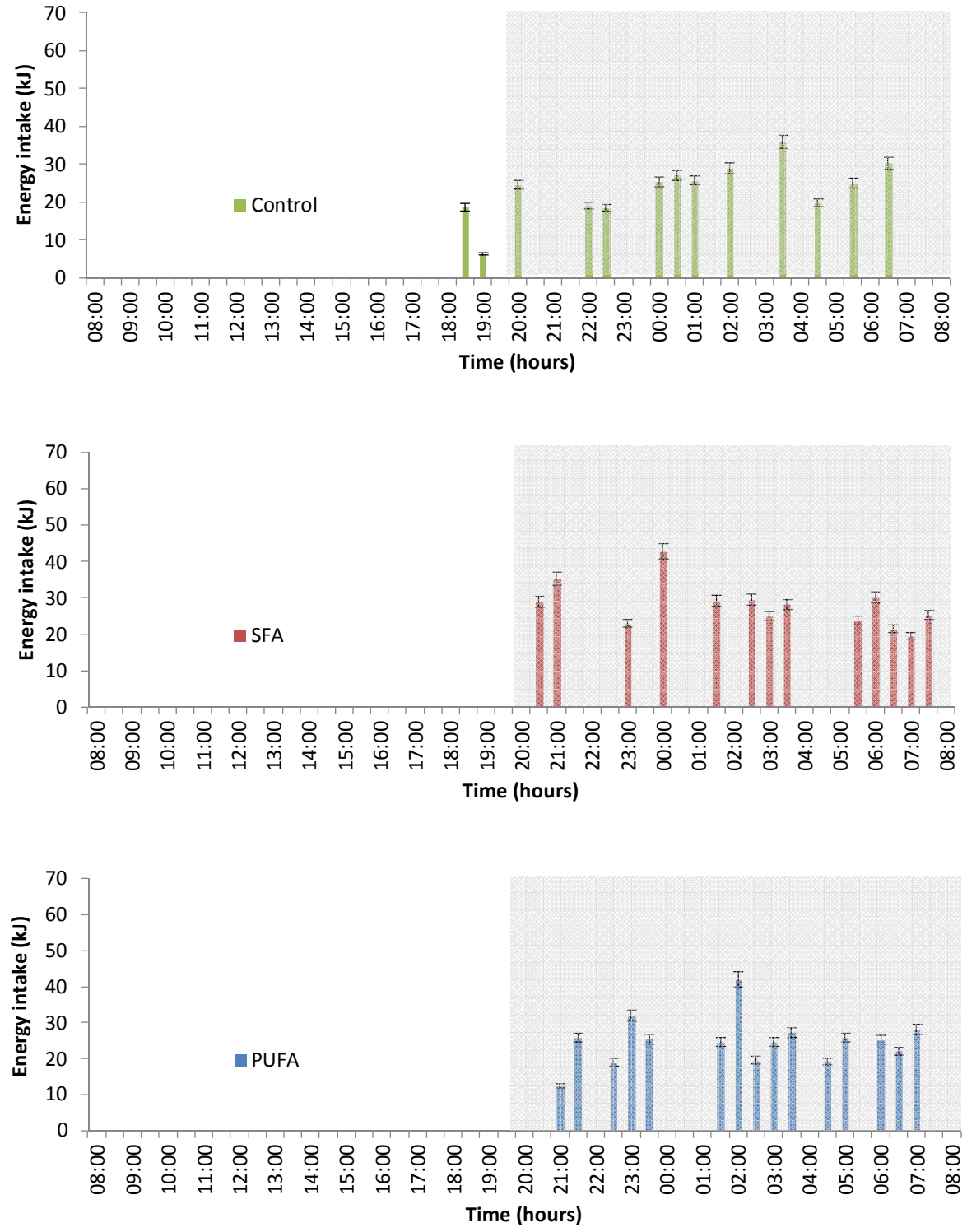



**Figure 3.13.** Circadian rhythmicity of feeding activity at one (A), three (B), five (C) and seven (D) weeks of consumption in control, SFA- and PUFA-fed rats. Data are presented as means of 30-minute intervals over 72 hours. Values are expressed as mean ± SEM (*n*=4/group). Grey shading represents the nocturnal phase.

**B.**


Energy intake (kJ)
Control
SFA
PUFA
Time (hours)
0
10
20
30
40
50
60
70
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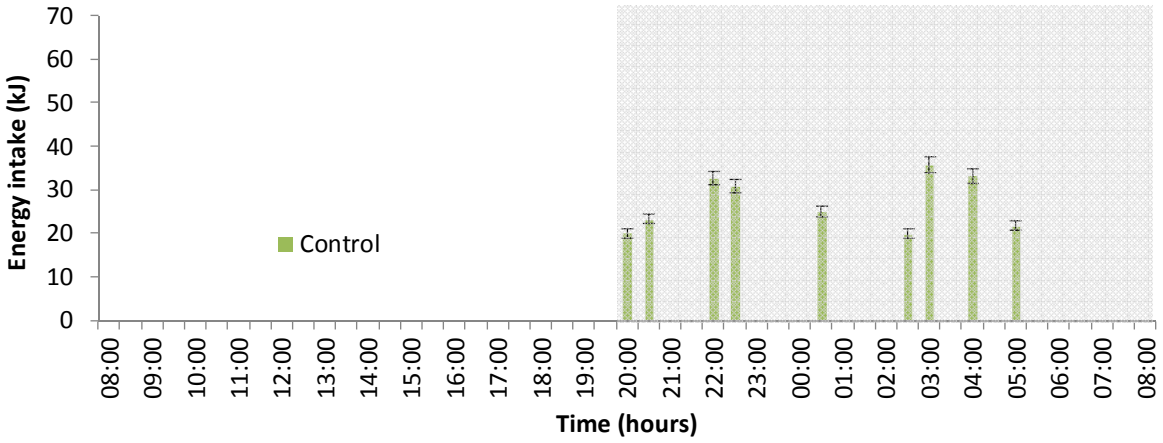
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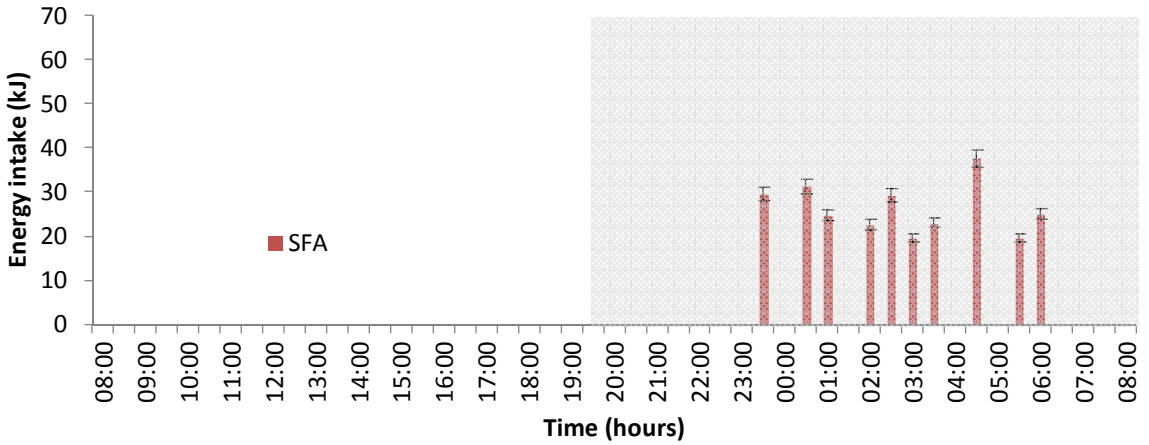
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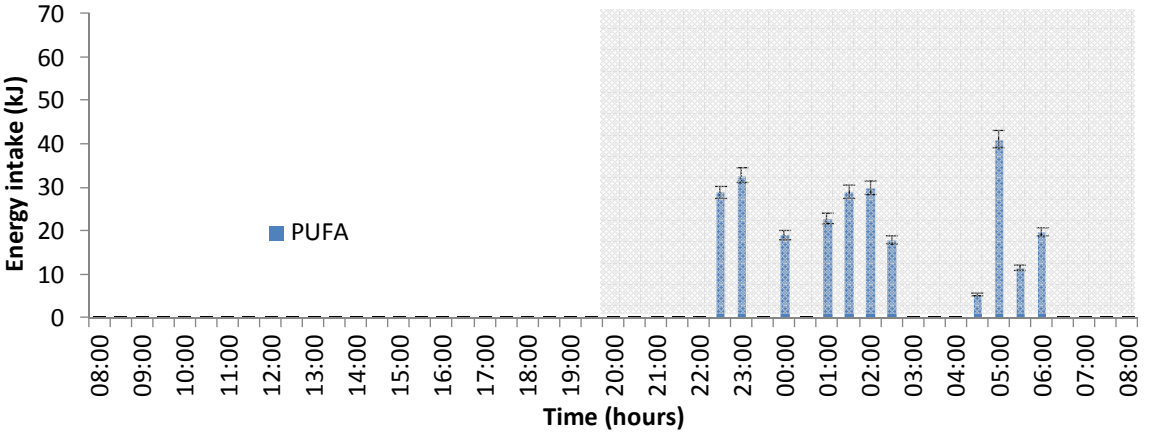
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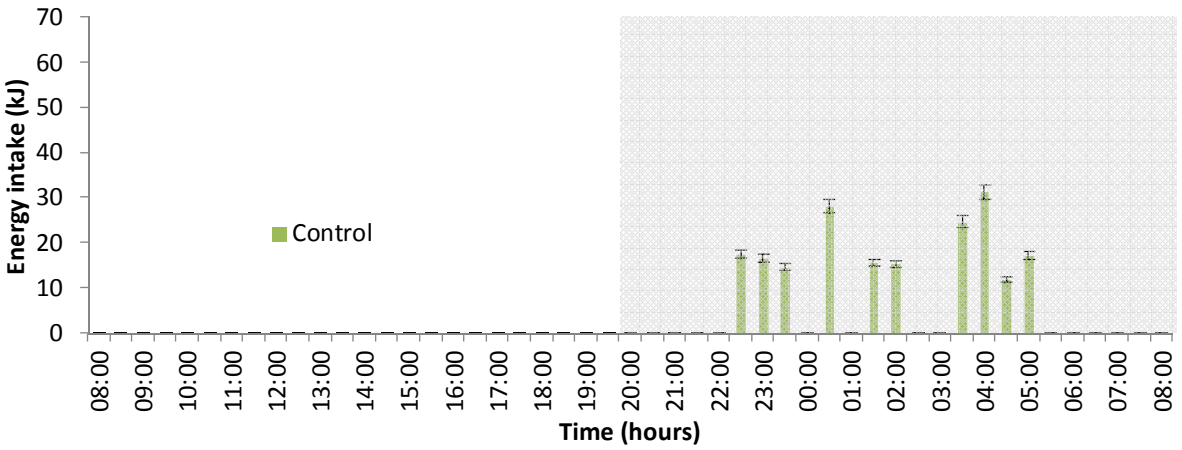
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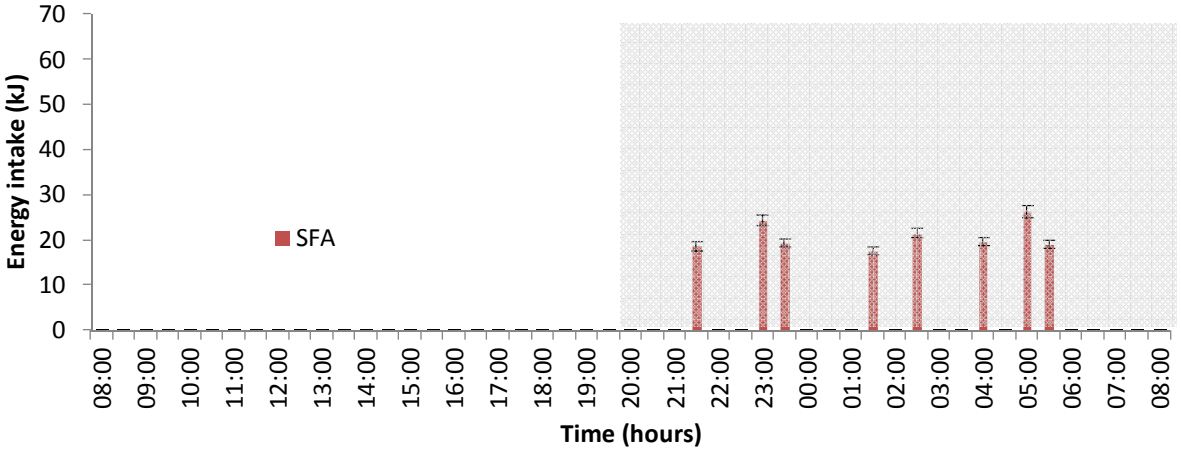
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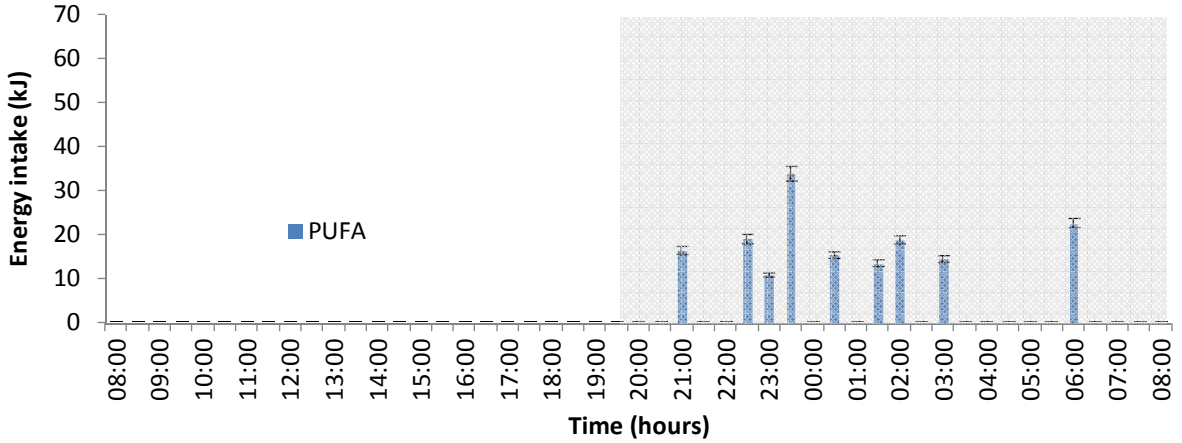
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Time (hours)

## 3.4 Discussion

To briefly summarise, there were few effects of diet on parameters of energy metabolism in this study. However, independently of an otherwise apparently obese phenotype, lipid metabolism and feeding behaviour were improved by high-PUFA intake.

While this study was underway, the ongoing overlap of food intake data collected from both high-fat fed groups was revealed. At first this was considered fortuitous, as it would have meant that any differences observed would be attributable solely to fat composition. However, the overlap observed later, on most other outcomes, raised the question of how much the two diets actually differed in composition. Closer examination revealed the presence of SFAs in the PUFA-enriched diet, and *vice versa* (Table 3.1), which could have offset or overridden the other's effects. Almost a third of the energy source from fat in the PUFA-enriched diet (~30%) was derived from SFAs present in the menhaden fish oil which also acted as the main PUFA source. Furthermore, almost a fifth of the energy source from fat in the SFA-enriched diet (~20%) was derived from PUFAs present in the lard, which also acted as the main SFA source (Appendices I and II). Therefore, it was clear that diets containing purer sources of SFAs and PUFAs would be required for future modelling of their individual effects. Findings are discussed in light of this realisation, and often in the general context of high-fat effects, irrespective of type.

### 3.4.1 Energy Intake, Body Weight & Adiposity

The equivalent, albeit transient, hyperphagia seen in both the PUFA- and SFA-fed groups compared to controls, unsurprisingly resulted in equivalent body weight gain. This suggests that provision of 40% of energy content as fat is sufficient to disturb appetite regulation, but that the type of fat has no effect. This is supported by some previous studies, where composition of dietary FAs has been shown to have little effect on body weight in rodents (Awad *et al*., 1990; Hill *et al*., 1992; Hill *et al*., 1993). In others, when dietary fats have been shown to differentially affect body weight, this has been overridden by total fat intake (Buettner *et al.,* 2006). Furthermore, diets containing approximately 40% energy as fat have been shown to induce obesity without stimulating hyperphagia, through elevated food efficiency (Oscai *et al.,* 1984). Although in the current study, food efficiency was similar in

both PUFA- and SFA-fed groups and reduced below that of low-fat-fed controls, when one considers the transience of the observed hyperphagia and the increased adiposity in both high-fat-fed groups, it could be argued that obesity had been effectively induced in the absence of hyperphagia. In addition, the amount of energy consumed decreased as the study progressed. This is typically observed in studies of this kind, and is most likely due to habituation, or adaptation, to the diet (e.g. Wood & Reid, 1975; Gajda *et al.,* 2007). Rodents consuming a diet low in fat will begin to eat less when switched to a high-fat diet, as they tend to regulate their energy intake, rather than grams of food consumed (Ricci & Ulman, 2008).

Surprisingly, the PUFA- and SFA-fed rats gained no more overall body weight than the control group, despite consuming more energy overall. The increased cumulative intake measure takes into account the transient increase within the first few weeks of dietary exposure, which may not have lasted long enough to permanently affect body weight. It is perplexing; however, that increased adiposity in these two groups did not lead to increased weight gain. It is possible that although there was fat accrual in the depots examined, there may have been fat loss in other depots not examined here, which offset this; i.e. an overall redistribution of body fat (Santosa & Jensen, 2008). Scanning the animals with Dual-energy X-ray absorptiometry (DEXA) can be used to measure total body composition and fat content with a high degree of accuracy. Whole-body composition measures, for example, by bioimpedance scanning, may also have provided a more accurate assessment of overall adiposity, and in light of this, was carried out in the following study (Chapter 5).

The lack of clear separation between control and experimental animals on this outcome (as well as others discussed below) was likely due to the presence of sucrose in the control diet (10% wt/wt). Studies have shown that feeding Wistar rats a diet containing elevated quantities of sucrose (63% wt/wt) causes a significant increase in body weight, specifically through increased epididymal fat pad mass (Soria *et al.,* 2001). Foods high in sugars (refined carbohydrates) stimulate weight gain by a number of means: stimulation of appetite through enhanced palatability; to an extent, passive overconsumption through their higher energy density; and ready conversion to triglyceride, storage in adipose tissue and growth in fat cell size (Tsujita *et al.,* 1995; Berger & Barnard, 1999; Dam & Seidel, 2007). Therefore, it is

clear that a more appropriate control diet, which did not contain sucrose, would be required for future modelling studies.

Composition of dietary FAs has been shown to affect distribution of adipose tissue differentially (Hill *et al*., 1992; Hill *et al.,* 1993). SFAs will increase whereas PUFAs will decrease WAT mass (Belzung *et al.,* 1993; Hainault *et al.,* 1993; Hill *et al.,* 1993; Baillie *et al*., 1999). Consistent with this, SFA-fed rats in the current study showed increased fat mass. So did the PUFA-fed group, probably due to the presence of SFAs in this diet. As there was no effect of diet on muscle mass, the increase in body composition ratio in high-fat diet groups reflected this increase in adiposity alone. This alteration in body composition by high-fat diets has been shown previously in rodent models fed diets providing one half or more of energy from SFAs (Hegarty *et al.,* 2002; Dourmashkin *et al*., 2005). In contrast, there was no effect of diet on BAT mass. BAT hypertrophy has been shown previously in rodents fed high-fat and cafeteria-style diets and has been associated with increased thermogenesis (diet-induced thermogenesis). It has been suggested that the additional energy intake is partially dissipated by BAT in an attempt to counteract obesity (Rothwell & Stock, 1979). However, this is subject to ongoing debate, as others have found that BAT is atrophied in diet-induced obesity (Muralidhara & Desautels, 1994). In addition, whether individual macronutrients or calories *per se* are responsible for this effect is the subject of ongoing research. In male Wistar rats fed diets ranging in fat content (17-93%), expression of genes involved in the regulation of thermogenesis increased accordingly, but only the diet with the highest content also increased BAT mass (through increased adipocyte size) (Betz *et al.,* 2012). It appears, therefore, that in the diets used here, provision of only 40% of energy as fat was insufficient to induce changes in BAT mass.

PUFA-fed rats also had enlarged livers which were paler in colour, suggesting an increase in fat deposition; this is consistent with the literature, as although PUFAs are incorporated into all cell membrane phospholipids, they are readily integrated into liver phospholipids, increasing the overall mass of the organ (Hill *et al*., 1993; Rapoport *et al*., 2010). PUFAs suppress hepatic lipogenesis and triglyceride synthesis, while inducing peroxisomal fatty acid oxidation (Clarke & Jump., 1994; Rambjor *et al*., 1996; Ren *et al.,* 1996; Akinori *et al.,* 2012). These effects on lipid metabolism are due to changes in gene expression leading to

induction or suppression of genes encoding key metabolic enzymes (Ntambi, 1992; Jump *et al.,* 1993; 1994). While the molecular mediators for PUFA regulation of these genes have not been defined, studies on the regulation of peroxisomal enzymes suggest that fatty acids activate a nuclear receptor to control gene transcription (Kliewer *et al.,* 1994; Braissant *et al.,* 1996; Schoonjans *et al*., 1996).

### 3.4.2 Plasma Leptin Concentrations & Adipose Leptin Gene Expression

The hyperleptinaemia displayed in both high-fat-fed groups is consistent with their equivalent fat masses, as leptin is expressed in proportion to the amount of body fat (Schwartz & Seeley, 1997). This relationship has been observed previously in rats fed SFA- and PUFA-enriched diets containing 40% of energy from fat (Reseland *et al.,* 2001). In contrast, others have shown that the consumption of PUFA-enriched diets elevates plasma concentrations of leptin above those in rats consuming high-SFA diets (Cha and Jones, 1998). However, discrepancies between this study and the one described here could be due to differences in diet composition, where energy from fat was half that of the current study and the PUFA source was mixed. Feeding patterns also differed as did the rat strain (Sprague-Dawley). Alternatively, these effects of PUFAs in the current study may have been overridden by those of SFAs present in the high-PUFA diet. Elevated concentrations of leptin in the blood should lead to a satiated state and maintenance of a normal body weight. However, both high-fat-fed groups gained in adiposity. This combined with the sustained elevation of leptin concentrations suggest that leptin resistance had been induced. This has been observed previously in rat in response to both types of high-fat diet (Lin *et al.,* 2000; Oh-I *et al*., 2005).

The equivalent plasma leptin concentrations observed in the high-fat-fed groups were not reflected in equivalent gene expression. The PUFA-fed group showed reduced leptin gene expression in all adipose depots, significantly so in the epididymal and inguinal depots. This at first seems paradoxical, given the confounding presence of SFAs in the PUFA diet. However, it has been observed previously, again in the work of Reseland *et al.* (2001), who also found that dietary PUFAs providing 40% of energy decreased leptin gene expression in rat epididymal adipose tissue. Nevertheless, observed changes were below one relative fold change and therefore unlikely to be of biological relevance (Glick & Pasternak, 2003; Logan

*et al.,* 2009). Measurements taken at the gene level and protein level rarely correlate; this is because of differences in post-transcriptional modifications and regulations. The impact of these mechanisms on the propagation of co-expression from gene to protein levels has not yet been systematically investigated. The dynamics introduce a time delay, since the corresponding molecular processes are not instantaneous (Anderson & Seilhamer, 1997; Tian *et al*. 2004; Cox *et al.,* 2005; de Sousa Abreu *et al*., 2009; Wang *et al.,* 2010). Studies have shown that leptin gene expression varies across different adipose depots, but whether this contributed markedly to observed differences in plasma leptin concentrations was uncertain (Rayner & Trayhurn, 1996). Others have also suggested that there is no link between leptin gene expression and circulating plasma concentrations (Ranganathan *et al*., 1998).

### 3.4.3 Plasma Adiponectin Concentrations & Adipose Adiponectin Gene Expression

Plasma adiponectin concentrations remained unchanged in both high-fat diet groups for the duration of the study. This is inconsistent with the observed increase in body weight and the increased fat mass also seen in these rats, as adiponectin is secreted from adipose tissue in concentrations inversely proportional to adiposity (Hu *et al.*, 1996). These very similar circulating concentrations in the high-fat groups were consistent with their statistically equal fat pad masses and gene expression in the WAT depots examined. This is in contrast to previous research in which partial replacement of SFAs with marine (omega-3) PUFAs in a high-fat diet increased adiponectin gene expression in the epididymal and perirenal fat pads of mice. A diet containing PUFAs is thought to increase both circulating adiponectin concentrations and adipose tissue gene expression due to a reduction in adiposity (Flachs *et al.,* 2006).

### 3.4.4 Whole-Blood Glucose & Plasma Insulin Concentrations

Fasting whole-blood glucose concentrations did not alter in response to either high-fat dietary intervention. Although diets high in SFAs can increase blood glucose concentrations, a mechanism has not been proposed (Briaud *et al.,* 2002), and in contrast, other studies modelling diet-induced obesity in rat have shown consistent normoglycaemia (e.g. Pickavance *et al.,* 1999; 2001; 2005). Also supporting the current results, it has been shown that PUFA supplementation does not affect glucose

concentrations (Rapoport *et al.,* 2010). Over the course of the study, plasma insulin concentrations continued to rise above baseline in all three diet groups, and significantly above control concentrations in both high-fat-fed groups, suggesting the development of insulin resistance (Ravussin *et al*., 2002). These results are consistent with the observed increase in body weight over time, as insulin is adipogenic. Stable, baseline glucose concentrations and the observed hyperinsulinaemia together suggest the rats were in a state of impaired glucose tolerance, which can prevail for many years in humans before the onset of frank diabetes (Ferrannini *et al.,* 2004). β-cell failure, leading to hyperglycaemia and overt diabetes are rarely, if ever, observed in DIO models (Levin *et al.,* 1997; Pickavance *et al.,* 2001; Rossmeisl *et al.,* 2003; Collins *et al.,* 2004; Chatzigeorgiou *et al.,* 2009; Peyot *et al.,* 2010). The differences observed here in circulating concentrations of insulin between rats fed the high-fat and control diets could, in part, be due to the increased adiposity of the high-fat-fed groups. Human studies have shown that consumption of diets high in fat cause weight gain, specifically through increased fat storage in and around abdominal organs (Isganaitis & Lustig, 2005). This is mirrored by the increased epididymal and perirenal fat pad masses observed in the rats in the current study. It is well-established that abdominal obesity is associated with insulin resistance (Gray *et al*., 1993; Pasquali & Vicemati, 2000; Catalano *et al.,* 2005) and that dietary fatty acids *per se* are implicated in its cause. Indeed, insulin resistance has been observed in high-fat-fed rats after just three weeks' consumption (Kraegen *et al*., 1991) and before a measurable increase in adiposity (Storlein *et al.,* 1991). Thus, consumption of SFAs, such as those found in lard, seems to be an effective way of producing insulin resistance in animal models (Storlien *et al.,* 1991). However, this can also be achieved by feeding rodents diets high in sucrose (Gajda *et al.,* 2007) and probably explains the progressive rise in circulating insulin in the control group. This undesired effect would be avoided in future, again, by designing a diet formulation without sucrose. Indeed, the sucrose in the high-PUFA diet may have exacerbated the insulin resistance induced by the SFAs also present in it. This is suggested by previous work in which menhaden fish oil could not prevent insulin resistance in sucrose-fed rats (Podolin *et al.,* 1998).

Although fish oil PUFAs are known to lower insulin concentrations in rat (Storlien *et al.,* 1987; Storlein *et al*., 1991) and are thought to explain the low rates of

insulin resistance, diabetes, and obesity that occur in individuals living predominantly on a fish-based diet (Ebbesson *et al*., 1999), their lack of effect here is likely due, once again to being superseded by SFAs present in the diet.

### 3.4.5 Plasma Triglyceride Concentrations

Although the PUFA- and SFA-fed rats were phenotypically almost identical, PUFA-feeding markedly reduced plasma TG concentrations below normal following only two weeks of consumption, an effect which was sustained throughout the study. This is a robust property of PUFAs and one of the known benefits of their consumption (Buckley & Howe 2009), particularly in the treatment of cardiovascular disease associated with hypertriglyceridaemia (Reseland *et al.,* 2001).

TGs act as transporters of dietary fat in the circulatory system. Lipoprotein lipase (LPL), found in vascular endothelium, is responsible for hydrolysing circulating TGs into glycerol and FAs. It is these free FAs and circulating TG that enable a dynamic equilibrium to exist between fat oxidation and fat storage in animals when consuming a normal diet (Scharrer, 1999). However, when a diet high in SFAs is consumed, changes in hypothalamic-pituitary-adrenal activity result in elevated glucocorticoid concentrations, which can lead to hypertriglyceridaemia through a reduction in LPL concentrations (Ghibaudi, *et al.* 2002). In contrast, when a diet high in fish oil is consumed, very-low-density lipoproteins, which enable fat and cholesterol to be transported within the bloodstream, can be decreased, which ultimately results in a reduction in plasma triglycerides (Lin *et al.,* 2005). The mechanisms behind this are thought to involve decreased hepatic triglyceride synthesis and more rapid oxidation of PUFAs than SFAs (Lin *et al.,* 2005).

Despite increased energy intake and adiposity in the SFA-fed group, triglyceride concentrations did not increase as expected. This may be due to the observed increase in insulin concentrations, as increased insulin secretion will promote storage of triglycerides in adipocytes, reducing circulating concentrations (Nishino *et al.,* 2007). Indeed, the partial reduction in TG levels in the SFA-fed group may be explained by the presence of PUFAs in that diet. Thus, in both high-fat-fed groups, dietary PUFAs and high levels of insulin may have acted in combination to prevent hypertriglyceridaemia.

### 3.4.6 Meal Patterns & Circadian Rhythmicity

The high-fat diets did not disrupt the temporal sequence of feeding (diurnal-nocturnal partitioning). With all diets, diurnal meals were consistently smaller and less frequent than nocturnal ones, which was to be expected, as rats are well-known for their nocturnal feeding activity (Strubbe & Woods, 2004). Although nocturnal feeding frequency decreased over the course of the study, in that rats took progressively fewer meals, the rate of feeding at each meal increased, while meal duration remained unchanged. This suggests that, as if to compensate for feeding less often, rats increased the amount they ate at each visit to the hopper, rather than increasing the amount of time spent eating, but not enough to prevent an overall reduction in food intake. The constancy of meal lengths also corresponds to unchanging glucose concentrations across the study in all groups, as blood glucose levels correlate with hunger, determining the start and termination of meals (Strubbe & Woods, 2004). Studies of rat models of both diet-induced and monogenic forms of obesity mirror findings in humans that obesity correlates directly with meal size, but inversely with meal frequency (Hariri & Thibault, 2010; Hinney *et al.,* 2010). Specifically, consumption of high-fat diets over a five-month period have been shown to lead to hyperphagia in male outbred rats through an increase in meal size, not number (Farley *et al.,* 2003). Although this pattern was shown in the high-fat-fed groups in the current study, its manifestation in the control group as well suggest that, at least in terms of influencing feeding behaviour, the diet compositions were not sufficiently different, such that, at the given concentrations, sucrose and fatty acids have similar effects. It is also possible that in only two months, group differences had not had time to develop.

Although the high-fat diets appeared to have little measurable impact on most aspects of feeding activity, importantly, they were distinguished by their effects on satiety. This is to be expected, as FAs are known to be metabolised differently depending on their degree of saturation, which differentially affects satiety in humans (Blundell *et al.*, 1993; Blundell & Macdiarmid, 1997; Fernandez-Quintela *et al.*, 2007). The enhancement of high-PUFA feeding in daytime satiety over the course of the study substantiates findings on PUFA-induced satiety in humans (Lawton *et al.,* 2000); for example, when consumed for three months, omega-3 FAs will suppress appetite in humans, and the mechanism of this action is thought to

occur through the associated elevation of satiety signal levels (Scharrer, 1999). However, the novelty of the current result means that there are no previous findings to explain the significance of enhanced satiety occurring during the day, rather than the night. The circadian effects of high-SFA diets have been examined, on the other hand, but findings are inconsistent. In some studies, high-SFA diets have been shown to increase food intake in rodents during the diurnal phase (Kohsaka *et al.,* 2007; Kaneko *et al.,* 2009), but to be less effective in producing satiety at the end of a feeding cycle (Geliebter, 1979; Walls *et al.,* 1992). This is thought to affect metabolism, leading to obesity (Arble *et al.,* 2009). Thus, it is unclear how the PUFA-induced satiety can coexist with the observed increase in adiposity, without being balanced, for example, by a reduced satiety at night. This is, in fact, what was observed (Figs. 3.12B, D & F), but again, only slightly below that of SFA-fed animals, possibly due, once again, to the SFA content in the PUFA diet. In other studies, rodents consuming diets very high in SFAs (67% energy from fat) consume fewer meals in the day than controls, consistent with increased IMI and satiety ratio (Hariri & Thibault, 2010). This, too, was observed here, but only subtly. What is perhaps most striking overall when considering the three diets together is that, although the shapes of the IMI and satiety ratio curves (Figs. 3.12A-D), and therefore, the progress of change in satiety differs, they all converge at the end of the study, supporting, once again, the idea of adaptation to the high-fat diets.

The rat consumes 90% of its energy intake in several discrete meals throughout the nocturnal phase (Armstrong, 1980; LeMagnen, 1981), with peaks occurring towards the beginning and end of this period (Siegal, 1961). This pattern alters in rats fed diets very high in fat (86% of energy provided as lard), as meals tend to shift towards the latter part of the dark phase (Tempel *et al.,* 1985). Food ingested throughout the first half of the nocturnal phase may be utilised to fulfil any immediate energy requirements, as well as for lipogenesis, whereas feeding at the end of the nocturnal phase may be more anticipatory of the diurnal phase to follow, and therefore, serves to build energy reserves. Indeed, the rats' stomach contains large amounts of undigested food at the end of the nocturnal phase (Armstrong *et al.,* 1978). Therefore, a high energy meal may be consumed at the end of the nocturnal phase in preparation for the 12-hour diurnal period when little feeding activity occurs (Tempel *et al.,* 1989). This shift was not observed here, but this could be due to the

more moderate fat content. Moreover, the characteristic peaks described above, towards 'lights on' and 'lights off', were not obvious in the control group either, again casting doubt on the validity of the control diet and suggesting that normal feeding periodicity may not actually have been observed in this study. Finally, the temporary clustering of meals in the middle hours of the night in the high-fat-fed groups' mid-way through the study may have been instrumental in stimulating accumulation of fat mass. Early nocturnal meal skipping has recently been shown to alter the peripheral clock and increase lipogenesis in mice (Yoshida *et al.,* 2012).

## 3.5 Conclusion

Overall, there were few effects of diet on parameters of energy metabolism in this study, likely due to similar diet compositions. However, independently of an otherwise apparently obese phenotype, lipid metabolism and feeding behaviour were improved by high-PUFA intake. This suggests that providing 52% of fat source from fish oil, with almost half derived from omega-3 FAs (Table 3.1), is sufficient to improve energy metabolism, at least to a limited extent, overriding the effects of potentially damaging SFAs present in the same diet. The confounding impact of SFAs on other parameters indicates that current dietary advice regarding increasing consumption of oily fish (Ruxton, 2004) should be balanced against greater awareness of the mixed composition of some fish oils and the detrimental effects it may have on health. The extent of these effects could be clarified by identifying the FA composition of the fat pads in the PUFA-fed group, for example, by fatty acid methyl ester (FAME) analysis. If mainly composed of PUFAs, this would have very different implications for the health of the model (Buckley & Howe, 2010). Also, a diet providing 40% of energy from fat is extreme and unlikely to be accepted long-term by humans, even if proven entirely healthful. Therefore, future studies would have to involve the use of purer sources of FA types, to help distinguish effects more clearly, as well as an expanded range of FA concentrations. The first of these criteria was to be addressed by the next dietary study in the project (Chapter 5).

Aside from the overlap in diet compositions and the moderately high sucrose content in the control diet, the lack of statistical differences between groups on several parameters could have been due to the fact that the rats were an outbred

strain. Such strains have inherent genetic variation (Koolhass, 2010) resulting from random DNA mutations. Laboratory rodent colonies are highly susceptible to genetic drift, as DNA variation often remains hidden at the molecular level and may be perpetuated by breeder selection processes. The result is a minefield of genetic variation which can confound research interpretation and results (Charles River, Margate, UK); the model in question is gradually altered, such that the response to a particular diet, for example, becomes less predictable (L.C. Pickavance, personal communication). However, given that common human obesity is believed to occur on a polygenic background, it was this very variation which could be argued to make outbred strains better representatives of human obesity than isogenic strains. It is also possible, with respect to the analysis of meal patterns in this study, that these experiments were underpowered ($n$=4/group), as behavioural studies, including those examining feeding, are known for high levels of variation, and the sample size is usually high (e.g. $n$=12/group; Farley *et al.,* 2003). Therefore, although logistically and financially challenging, future studies on the determination of meal pattern signatures, with a limited set of automated cages, should be designed to accommodate larger sample sizes wherever possible.

# CHAPTER 4

## EFFECTS OF CHRONIC INTAKE OF DIETARY FATTY ACIDS ON SKELETAL MUSCLE FIBRE COMPOSITION

# Effects of Chronic Intake of Dietary Fatty Acids on Skeletal Muscle Fibre Composition

## 4.1 Introduction

### 4.1.1 Effects of Obesity and Dietary SFAs on Muscle

As reviewed in the General Introduction (Chapter 1, Section 1.2), the detrimental effects on health due to long-term consumption of saturated fatty acids (SFAs) are well-established, and include the development of obesity and associated conditions (Blundel *et al*., 1993; Blundell & Macdiarmid, 1997). Particularly in view of the fact that there is no pharmaceutical intervention for obesity which has proven both safe and effective in the long-term, many experts still maintain that lifestyle interventions, including increased exercise, are as effective, if not more so (Fujioka *et al.,* 2000). Energy expenditure, through sustained exercise, depends on the aerobic capacity of muscle, and can help reverse some of these detrimental effects (Ripple & Hess, 1998; Strømme & Høstmark, 2000).

Most muscles consist of a mixture of different fibre-types, depending on the function of the whole muscle, which are categorized as Type I or Type II. Type I fibres are suited to endurance activities and are slow to fatigue because they use oxidative metabolism to generate ATP. Type II fibres are efficient for short bursts of speed and power, as they are quicker to fatigue and use both oxidative metabolism and anaerobic metabolism. Muscles which enable aerobic exercise are "fat-burning" and fatigue-resistant, and so are richer in the slow-twitch, lipid-oxidative fibre-type (Type I) than the fast-twitch, glycolytic type (Type II) (Peter *et al.,* 1972). There is evidence from both animal and human studies that obesity is associated with altered fibre-type proportions. In comparison to their lean counterparts, genetically obese (*ob/ob*) mice show fewer fast-twitch (Type II), in relation to slow-twitch (Type I) fibres, in both soleus and extensor digitorum longus (EDL) muscles (Warmington *et al.,* 2000). Consistent with this slower-type profile, these muscles also show an increased fatigue resistance. Although this may at first seem counterintuitive, it could be that these features reflect a response to the inactivity associated with the obesity. Indeed, these muscles show reduced mass, resulting from lack of use (atrophy) and a reduced ability to hypertrophy (Warmington *et al.,* 2000).

The effects of diet-induced obesity on the relationship between muscle form and function appear to vary. Rodent studies have shown that SFAs impair the ability of muscle to oxidize lipid (Sparks *et al.,* 2005) by impairing mitochondrial capacity for fat oxidation (Holloszy, 2009), and that they can do so after only very short periods of consumption (e.g. 3-15 days) (Sparks *et al.,* 2005). In general, this alteration in muscle function also appears to arise from changes in morphology. For example, mice with obesity induced by high-fat feeding (60% energy from fat for 8 weeks) ultimately showed reduced oxidative capacity in muscle, despite early adaptation to diet reflected in increased numbers of Type I fibres and fewer Type II fibres. This occurred in gastrocnemius, but not tibialis muscle, and was thought to be insufficient to influence contractile properties (Shortreed *et al.,* 2009). Human subjects sensitive to an obesity-inducing diet, as defined by resistance to weight loss, have also been shown to develop an increase in Type I fibres, thought to be an adaptive response to obesity (Gerrits *et al.,* 2010). Other human studies have shown the opposite, a reduction in Type I fibres associated with obesity-related variables, such as increased body fat percentage and waist-to-hip ratio (Karjalainen *et al.,* 2006). Conversely, increased muscle mass in transgenic mice, due to relative abundance of Type II fibres, is associated with regression of obesity induced by a high-fat, high-sucrose diet (Izumiya *et al.,* 2008). Findings in rat appear somewhat contradictory, however. Although muscle expression of genes encoding fat oxidative enzymes was shown to increase in obesity-prone Wistar rats fed a high-fat diet (40% energy from fat for 5 weeks), it was not accompanied by a change in fibre composition in gastrocnemius, soleus or EDL muscles (Zou *et al.,* 2003). In contrast, this gene expression was unaltered in the soleus muscle of Sprague-Dawley rats fed for the same length of time on a diet providing almost twice as much energy from fat (78%; McAinch *et al.,* 2003). Further confounding is the discovery that Wistar rats resistant to obesity, induced in susceptible counterparts by 4 weeks' consumption of diet providing 60% of energy from fat, showed an increased proportion of Type I fibres in gastrocnemius muscle; and this was thought to be linked to the increased fatty acid oxidation capacity which favours resistance to body fat accumulation (Abou Mrad *et al.,* 1992).

Thus, although fibre-type profiles in diet-induced obesity are variable, it is probably safe to say that, in general, SFA consumption stimulates a change in fibre-

type profile which is both time-dependent and muscle-specific, but where anatomy and function do not always relate. The differences in both dietary SFA concentration and duration of exposure across studies may also account for their discrepancies. It could be cautiously proposed that an initial, adaptive response to the presence of SFAs occurs to prevent obesity, one which stimulates fat oxidation and involves a transition to a slow fibre-type profile. However, with sustained exposure to SFAs, this response does not persist. If studies are conducted outside this early window, when obesity is established, it could explain observations of fast fibre-type profile and reduced capacity for sustained physical activity, and in turn, the increased likelihood of further weight gain.

### 4.1.2 Effects of Dietary PUFAs on Muscle

In contrast to the effects of SFAs, polyunsaturated fatty acids (PUFAs) in the diet may be beneficial to muscle function. Indeed, the shift toward overall improvement in metabolic function, which is induced by PUFAs, is partly achieved by upregulation of gene expression favouring fatty acid oxidation, including that within muscle tissue itself (Izumiya *et al.,* 2008; Shortreed *et al.,* 2009), and downregulation of expression which favours adipogenesis (Bordoni *et al.,* 2006). PUFAs also influence membrane-dependent activities, including those required for muscle function, through their incorporation into membrane phospholipids (Benatti *et al.,* 2004). In humans and birds, PUFA consumption has been shown to protect muscle function, stimulating energy metabolism and increasing the capacity for sustained exercise by improving fatigue-resistance (Maillet & Weber, 2006; Demmig-Adams & Carter, 2007). Therefore, there is indirect evidence that PUFAs increase the capacity of muscle for sustained exercise, but anatomical underpinning is required. Although some direct effects of dietary SFA composition on proportions of fibre-types have been examined, as discussed above, those of PUFAs are currently unknown.

In humans, it is thought that dietary PUFAs, consumed as fish oil supplements, act within skeletal muscle to reduce whole-body oxygen demand during extended periods of exercise (Peoples *et al*., 2008). Exercise capacity of patients suffering chronic obstructive pulmonary disease (COPD) has also been shown to increase after consuming a PUFA-enriched diet, due to increased oxidative

capacity in muscle (Broekhuizen *et al.*, 2005). In rats, the omega-6 and omega-3 FA profile of the diet determines phospholipid composition of muscle (Ayre & Hulbert, 1996), but how this may be reflected in fibre composition has not yet been examined. In general, however, these findings point to the replacement of dietary SFAs with PUFAs as a rational therapeutic approach to improving the metabolic profile and function of muscle (Demmig-Adams & Carter, 2007; Gillies, 2007). Increasing the capacity for fatigue-resistant muscle and therefore, allowing for sustained, extended, periods of exercise would aid obese individuals in targeting weight loss through increased physical activity.

Another question is what might cause the transition between predominating fibre-types, and this is partly addressed in Chapter 7. Muscle development results from the complex interaction of diet, genes, and exercise (Demmig-Adams & Carter, 2007), and its mechanism provides the rationale for FA-induced remodelling. Although different levels and types of exercise drive differential expression of muscle fibre-types (Howald *et al.,* 1985), the impact of diet is still not fully understood. Gene deletion and over-expression studies have shown that peroxisome proliferator-activated receptors (PPARs) regulate the synthesis of muscle fibres and induce changes in fibre composition (Ahmetov *et al.,* 2006; Schuler *et al.*, 2006). It has also been shown that dietary FAs act as PPAR ligands to regulate the expression of a variety of genes involved in nutrient metabolism (Kersten, 2002; Fernández-Quintela *et al*., 2007). Therefore, we might reasonably suppose that dietary FAs exert their effects on the synthesis and development of muscle *via* PPARs. This has been suggested in transgenic mice, where enhanced PPARγ activity has been shown to protect against the effects of high-fat feeding, including insulin resistance, and to increase the proportion of Type I fibres in muscle (Amin *et al.,* 2010). The supposition is contradicted, however, by the fact that very high-fat feeding (78% energy from fat) has no effect on PPARα or PPARγ protein expression in slow (soleus) or fast (EDL) muscles (McAinch *et al.,* 2003). Thus, more work is required to confirm the distinguishing effects of dietary FA composition on relationships between muscle form and function.

### 4.1.3 Research Question

***Do dietary fatty acid composition determine the fibre-type population of skeletal muscle? If so, do PUFAs induce a shift towards fatigue-resistant fibre-types?***

### 4.1.4 Aims & Expected Outcomes

To address these questions by

1. demonstrating that consumption of diets equally enriched in SFAs and PUFAs would induce different ratios of fast- to slow-twitch fibres in skeletal muscle of rat; and furthermore, that

2. high-PUFA consumption would result in a greater proportion of slow-twitch fibres.

Although the answer to this research question is valuable in its own right, there is an additional, implied purpose to this study, which is to explore further the effects of chronic PUFA consumption documented in Chapter 3:

### 4.1.5 Background

Dietary fatty acids differentially affect adipose tissue on several parameters [distribution, fatty acid composition, accrual (fat accumulation and adipocyte size)], as well as whole-body composition (fat-to-lean ratio) (Hill *et al.,* 1992, 1993; Gajda *et al.,* 2007; Barasi, 2008; Buckley & Howe, 2009).

### 4.1.6 Research Question

Thus, it is interesting to determine if this remodelling capacity of PUFAs extends to the functional microanatomy of other peripheral tissues, such as skeletal muscle.

## 4.2 Materials and Methods

### 4.2.1 Animals and Dietary Treatment

The analyses presented here were carried out on the same animals used in the study described in Chapter 3 (Section 3.2). Two groups of adult male Wistar rats (~ 250 g) were fed isoenergetic diets enriched with either SFAs or PUFAs for eight weeks. Both diets provided 40% of energy from fat, mainly from lard and fish oil, respectively ($n$=8/group). Details of general husbandry and maintenance can be found in Chapter 2, Section 2.1.6 and diet composition in Chapter 3 (Table 3.1). Due to financial and logistical constraints of the project, the low-fat control group ($n$=8/group) was run separately and muscle samples collected and stored for future analysis.

### 4.2.2 Tissue Collection and Processing

Gastrocnemius and soleus muscles were dissected fresh from rat cadaver hind limbs, as representative examples of anaerobic/glycolytic and aerobic/lipid-utilizing types, respectively, such that any alteration of fibre composition would be clearly observable. Histological processing was carried out according to protocols optimised in-house with Dr. I.S. Young (Institute of Integrative Biology, University of Liverpool). After immersion-fixation for three hours in 4% buffered paraformaldehyde, muscles were cryoprotected in 10% buffered sucrose. The belly of each muscle was blocked, mounted on a specimen holder in OCT compound (Fisher Scientific, Surrey, UK) and frozen at -20$^{o}$C within a cryostat (Leica CM1900, Leica Microsystems, GmbH, Germany). Transverse 10-µm sections (perpendicular to the long axis of the muscle) were collected 60 µm apart, thaw-mounted onto chrome-alum and gelatin-coated slides, air-dried and washed 3 x 10 minutes in 10 mM PBS.

### 4.2.3 Immunohistochemistry, Image Analysis and Quantification

To allow distinction between fast and slow fibre-types, sections were incubated for two hours at room temperature with monoclonal antibodies raised against adult human myosin isoforms (A4.74 and A4.840, markers of fast and slow fibres, respectively; Developmental Studies Hybridoma Bank, Iowa, USA). These antibodies show cross-reactivity with rat myosin and were applied at a dilution of

1:10. After washing, all sections then underwent a one-hour incubation, at room temperature in the dark, with Dylight 594-conjugated donkey anti-mouse IgG (1:500; Jackson ImmunoResearch Laboratories). After a final wash, all sections were air-dried and coverslips applied over VectaShield® anti-fade mounting medium (Vector Laboratories Inc., Burlingame, USA). General details of antibody dilution, incubation and wash steps are outlined in Chapter 2 Section 2.10.4. Immunofluorescent staining was examined under epi-fluorescence microscopy (Zeiss Axio Imager. M1). Images were captured by digital microphotography (Hamamatsu ORCA I-ER, Hamamatsu Photonics, Welwyn Garden City, Herts) and image analysis software (AxioVision, Carl Zeiss MicroImaging, GmbH, Germany).

Systematic counting was used to estimate fibre-type numbers in whole muscle cross sections taken from gastrocnemius and soleus muscles in rats fed high-SFA or high-PUFA diets for 8 weeks. All transverse muscle fibre profiles within each section were counted and categorised as stained or unstained respectively. Using image analysis software (Image J, National Institutes of Health, USA), counts were performed twice, on images photographed at 20x magnification, by two experimenters blind to the dietary treatment, muscle type or myosin isoform which had been stained (Abramoff *et al.,* 2004).

### 4.2.4 Statistics

Counts of fibre profiles with specific staining were expressed as a proportion of the total number of profiles (stained + unstained). Data are expressed as mean ± SEM. Differences in proportions of fibre-types between the two high-fat diet groups were determined using Student's 2-tailed *t*-test and were considered significant at $p<0.05$. Gastrocnemius and soleus muscles are known to consist of significantly different proportions of fibre-types (Peter *et al.,* 1972), and so it was not necessary to statistically compare results in response to this factor (muscle-type).

## 4.3 Results

Analysis of myosin immunohistochemistry (Figs. 4.1 & 4.2) revealed that, irrespective of diet, gastrocnemius and soleus muscles predictably showed inverse fibre compositions: more fast than slow fibres were present in the gastrocnemius muscle (mean across diets: 69±2.2% fast *vs.* 31±1.1% slow, $p<0.05$; Fig. 4.3). The opposite was true of the soleus muscle, and was more pronounced (12±0.8% fast *vs.* 88±2.8% slow, $p<0.05$; Fig. 4.3). No significant differences were found between diet effects in either muscle (both $p>0.05$). Mean fibre counts are presented in Table 4.1.

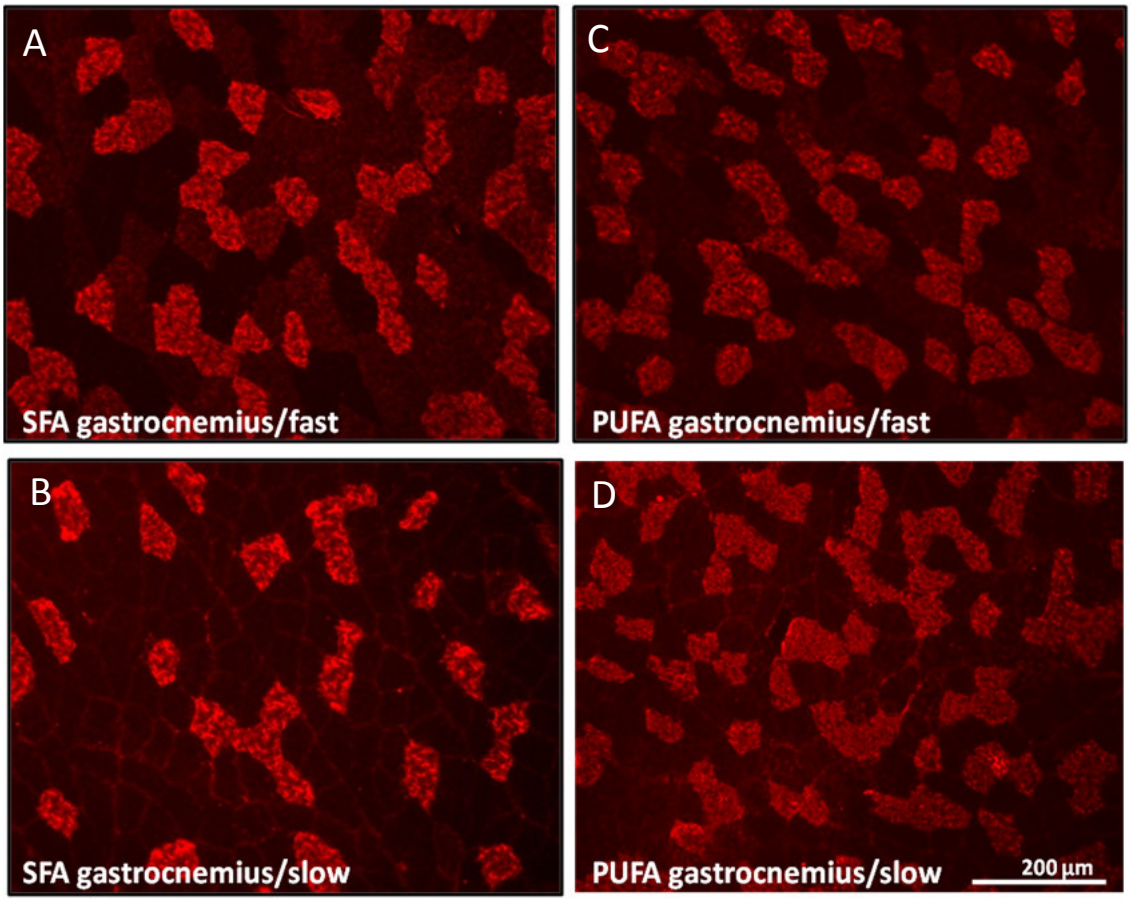


**Figure 4.1.** Representative photomicrographs of fast and slow fibres-types in transverse sections of **gastrocnemius muscle** from rats fed high-SFA (A, B) or high-PUFA diets (C, D) for 8 weeks ($n$=8/group). Stained fibres are indicated by red fluorescent angular profiles. Fibre-types were identified as fast (A, C) or slow (B, D), depending on the myosin isoform stained. *In this instance SFA-fed rats act as a control for PUFA-fed rats.*

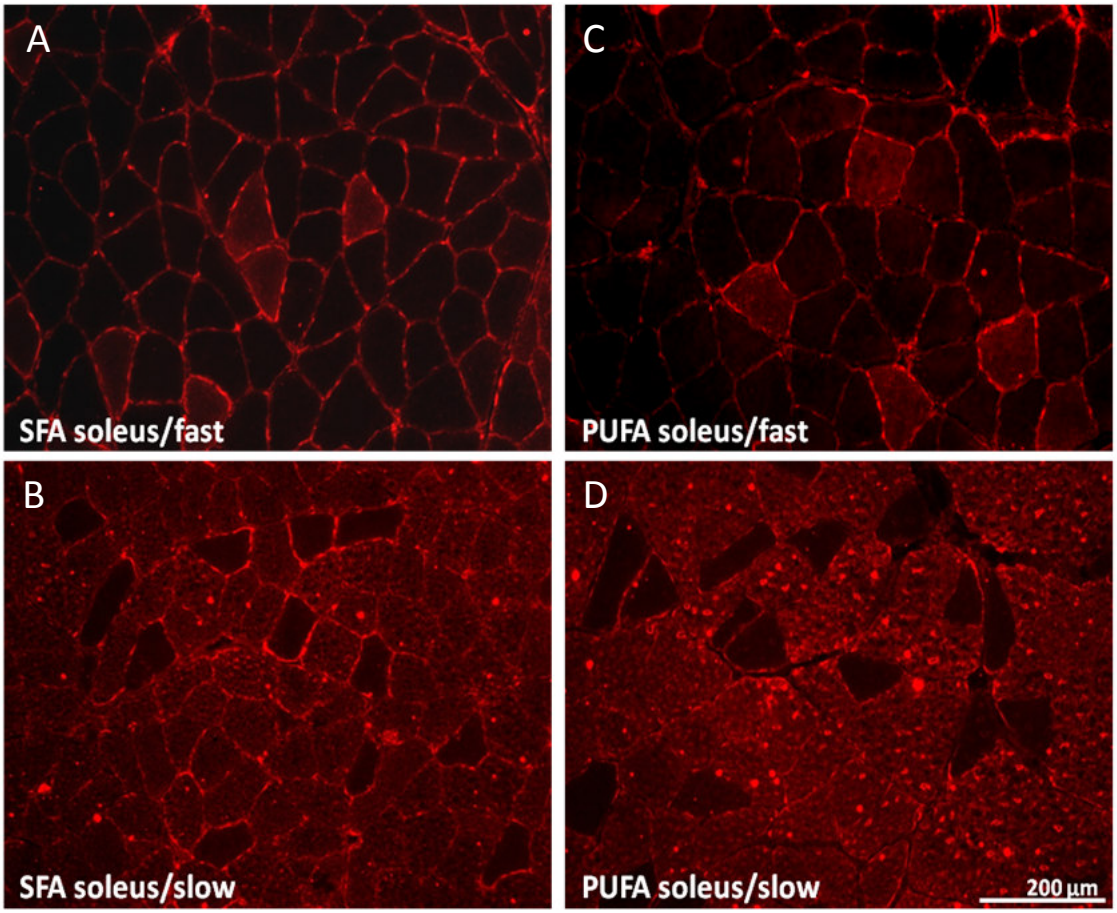


**Figure 4.2.** Representative photomicrographs of fast and slow fibre-types in transverse sections of **soleus muscle** from rats fed high-SFA (A, B) or high-PUFA diets (C, D) for 8 weeks (*n*=8/group). Stained fibres are indicated by red fluorescent angular profiles. Fibre-types were identified as fast (A, C) or slow (B, D), depending on the myosin isoform stained. *In this instance SFA-fed rats act as a control for PUFA-fed rats.*

**Table 4.1.** Total fibre-type counts in whole muscle cross sections taken from gastrocnemius and soleus muscles in rats fed high-SFA or high-PUFA diets for 8 weeks. All transverse muscle fibre profiles within each section were counted and categorised as stained or unstained respectively. Values are expressed as mean ± SEM ($n$=8/group): $p$>0.05 between diet groups. *In this instance SFA-fed rats act as a control for PUFA-fed rats.*

| | **Diet Type** | |
|---|---|---|
| **Muscle/Fibre Type** | **SFA** | **PUFA** |
| G –Fast | 42±4.8 | 47±6.2 |
| G – Slow | 29±6.5 | 30±3.7 |
| **G – Total** | **71±8.1** | **77±6.5** |
| S – Fast | 16±1.8 | 16.94± |
| S – Slow | 66±5.5 | 70.19± |
| **S - Total** | **82±7.8** | **86±5.4** |

**Abbreviations:** G = gastrocnemius, S = soleus.

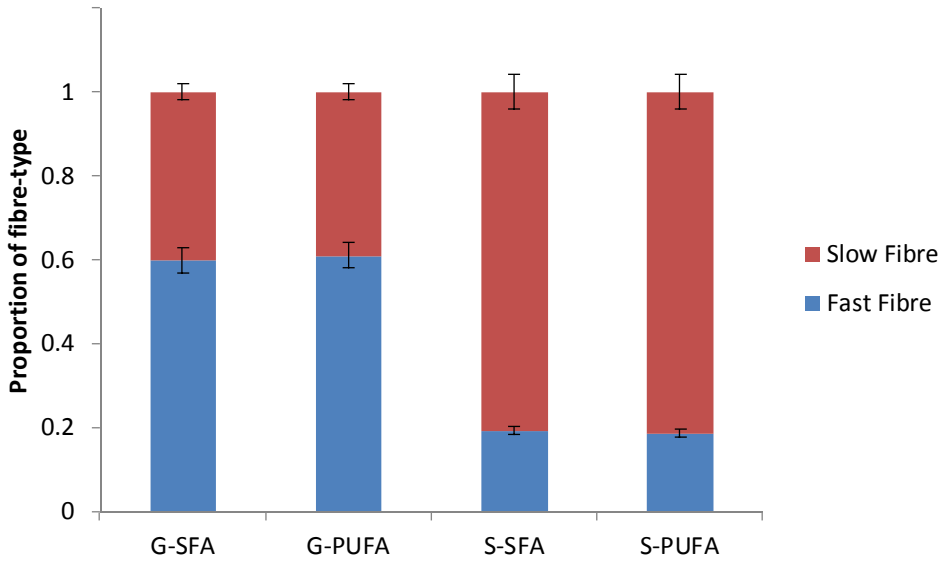


**Figure 4.3.** Fibre-type composition of gastrocnemius and soleus muscle in all rats fed high-SFA or high-PUFA diets for 8 weeks. Proportions were determined by quantification of total fibres immunostained for fast and slow myosin isoforms in all muscle cross sections collected (Figs. 4.1 and 4.2). Values are expressed as mean ± SEM ($n$=8/group): $p$>0.05 between diet groups. *In this instance SFA-fed rats act as a control for PUFA-fed rats.* **Abbreviations:** G = gastrocnemius, S = soleus.

## 4.4 Discussion

Results in Chapter 3 show that neither high-fat diet affected the mass of gastrocnemius nor soleus muscles compared to that of low-fat-fed control animals (Fig. 3.3D). This is consistent with the lack of effect of diet on the total number of fibres present in each muscle. The failure of the high-SFA diet to influence muscle mass contradicts findings in humans and animals, in which obesity has been associated with changes in the capacity of muscles to hypertrophy, although these findings themselves are contradictory. In *ob/ob* mice, atrophy and reduced ability to hypertrophy (Warmington *et al.,* 2000) is in contrast to the increased hypertrophy observed in human subjects sensitive to an obesity-inducing diet (Gerrits *et al.,* 2010). However, these discrepancies could be due to the properties of different muscles examined in the two studies (i.e. if they were not homologues), and/or to the differing etiologies of genetic and diet-induced obesity.

Neither were there different fibre-type proportions associated with the different high-fat diets, and therefore, no observed shift from fast- to slow- fibre-types induced by the high-PUFA diet. This is consistent with the overlap in several other outcomes presented in Chapter 3, including concentrations of circulating adipokines and insulin. Leptin replacement in *ob/ob* mice has been shown to shift the soleus muscle toward a fast fibre-type (Type II) profile (Warmington *et al.,* 2000). Therefore, the leptin resistance observed in the two high-fat-fed groups in the current study (Fig. 3.4) may provide some explanation for the lack of effect, although the anticipated effect of PUFAs was the opposite to this (a shift toward a slow fibre-type). Furthermore, in transgenic mice, enhanced PPARγ activity induces increases in both Type I fibre-type proportion and local secretion of adiponectin, which enhances muscle insulin sensitivity, suggesting there may be a relationship between all three features (Amin *et al.,* 2010). Again, the similar adiponectin levels and insulin resistance observed in the high-fat-fed groups in the current study (Figs. 3.6 & 3.9) are, therefore, consistent with similar fibre-type proportions.

As skeletal muscle composition is thought to result from the complex interaction of diet, genes, and exercise (Demmig-Adams & Carter, 2007), and as different levels and types of exercise drive differential expression of muscle fibre-types (Howald *et al.,* 1985), it could be proposed that exercise was the missing

stimulus for change in fibre-type proportions in the current study. Rats were sedentary and were not given opportunities to exercise, for example, by being provided with exercise wheels. However, this idea is challenged by a number of previous studies in which humans and rodents displayed altered muscle composition or oxidative gene expression in association with genetic or high-fat diet-induced obesity, despite the absence of exercise (Warmington *et al.,* 2000; McAinch *et al.,* 2003; Gerrits *et al.,* 2010).

It is also tempting to think that diet exposure may not have been long enough to induce change in muscle composition. This possibility is suggested by one other study, in which Wistar rats fed a high-fat diet providing the same amount of energy from fat as that of the current study (40%), but over a shorter period (5 weeks *vs.* 8 weeks here), showed no change in fibre composition in a range of skeletal muscles, including those examined here (gastrocnemius and soleus; Zou *et al.,* 2003). On the other hand, a number of other studies involving high-fat feeding for the same or shorter periods, and in which muscle composition or oxidative gene expression was altered, would dispute this (Abou Mrad *et al.,* 1992; McAinch *et al.,* 2003; Shortreed *et al.,* 2009). These latter studies, however, also involved the use of diets providing higher concentrations of energy from fat than those used here (60-78% *vs.* 40%), suggesting that this feature of the diet may be the critical one for altering fibre-type proportion. As discussed in Chapter 3, it would also seem entirely likely that the two high-fat diets used here failed to induce distinct fibre-type proportions because of their impure compositions.

Therefore, it has yet to be determined whether dietary PUFAs can remodel skeletal muscle by altering the ratio of fast-to-slow fibre-types, or if their beneficial effects on muscle function occur by another mechanism entirely (Broekhuizen *et al.*, 2005).

# CHAPTER 5

## CHARACTERISATION OF A REFINED PUFA-FEEDING MODEL

# Characterisation of a Refined PUFA-Feeding Model

## 5.1 Introduction

### 5.1.1 Rationale

The study described in Chapter 3 had aimed to develop a valid rat model of chronic high-PUFA consumption. Adult male Wistar rats fed a diet providing 40% of energy from PUFAs for two months were compared to controls fed an isoenergetic diet enriched with SFAs on a range of morphometric, metabolic and behavioural parameters. Compared to a low-fat-fed control group, they differed on only two measures, satiety and circulating concentrations of triglycerides. The otherwise extensive overlap between the outcomes of both types of dietary intervention was attributed to the presence of each FA type in the opposing diet. Furthermore, the incomplete separation between both high-fat diet and the control group on some parameters was attributed to the presence of sucrose in the control diet.

Thus, the original experimental aim had not been fully met, and it was considered that the model required refinement through re-design of diets. This entailed identification of PUFA and SFA sources with the least possible contamination by the other FA type. Thus, the study was designed to re-address the original aim, but while also taking account of financial constraints on the project which had arisen by this time, as well as limited availability of the chosen PUFA source. This meant that the diet exposure period, though not acute (as in only a few days long), was shorter than the previous one (3 *vs.* 8 weeks), and that concentrations of only a limited range of circulating factors could be examined. Those selected included triglycerides (TGs), to enable confirmation of the metabolic efficacy of the new high-PUFA diet, and brain-derived neurotrophic factor (BDNF), in an attempt to integrate findings with concurrent exploration of the diet's effects on cell proliferation in the brain.

### 5.1.2 Short-Term Exposure to PUFAs and Selection of Omega-3 Fatty Acid

The beneficial effects of PUFA consumption observed in the previous dietary study (Chapter 3; i.e. reduced plasma TGs and increased satiety) have been documented in humans (Buckley & Howe, 2009) and therefore, support the partial validity of that

model. These changes had occurred within the first two weeks of consumption. Short-term exposure to diets high in SFAs increases weight gain, but critically, impairs learning and cognition even prior to obesity onset (Kanoski & Davidson, 2011). In contrast, similar short periods of PUFA consumption show positive metabolic effects, such as attenuation of weight gain and decreased adiposity and circulating concentrations of TGs (Cunnane *et al.,* 1986; Belzung *et al.,* 1993; Hainault *et al.*, 1993; Baillie *et al.,* 1999; Ruzickova *et al*., 2004), and these health benefits have been attributed in particular to omega-3 FAs (Buckley & Howe, 2009). Therefore, a high-omega-3 source commercially purified from fish oil was considered an optimal replacement for the mixed-content fish oil used in the previous study (Chapter 3); docosahexaenoic acid (DHA) was selected because of its positive effects on health, both peripherally and centrally. For instance, it improves lipid metabolism, is required for normal nervous system (CNS) development, and its dietary supplementation is associated with improved cognitive health (Ruzickova *et al*., 2004; Buckley & Howe, 2009; Gomez-Pinilla, 2011; Poudyal *et al.,* 2011).

### 5.1.3 Obesity and BDNF

BDNF is a neurotrophin with diverse functions, ranging from development of the nervous system to the control of appetite (Chao *et al.,* 2006; Ooi *et al.,* 2012). Evidence from both human and rodent studies suggests that alterations in BDNF signaling, in appetite related regions of the brain, may be responsible for the increased food intake associated with obesity (Tapia-Arancibia *et al.* 2004; Levin 2007; Cordeira & Rios, 2011; Noble *et al.* 2011). BDNF and its receptor, TrkB, are currently under study to fully determine their functions, as they represent a potential target for the development of anti-obesity therapies (Hashimoto *et al.,* 2005; Ooi *et al.,* 2012). The dual role of BDNF is important in both the peripheral and central nervous systems, as it is key to aiding in proliferation, differentiation and survival of neurons, processes increasingly being found to underpin its requirement for sustaining behavioural processes, such as feeding (Cordeira & Rios, 2011; Noble *et al.* 2011). This latter role is investigated further in the study described in Chapter 7. In the CNS, BDNF is expressed at its highest concentrations in neurons of the hypothalamus, specifically, within regions related to feeding, such as the ventromedial hypothalamic (VMH) and paraventricular nuclei (PVN; Wu *et al.,* 2004a; Wu *et al.,* 2004b; Webster *et al.,* 2006; Godar *et al.,* 2011). It is known to

regulate food intake and energy expenditure through its actions in these two nuclei (Wu *et al.,* 2004a; Wu *et al.,* 2004b; Vaynman & Gomez-Pinilla, 2006; Godar *et al.,* 2011; Noble *et al.,* 2011; Rosas-Vargas *et al.,* 2011).

Indeed, leptin, insulin, glucagon-like peptide-1 (GLP-1) and other appetite regulators exert their effects through BDNF (Vaynman & Gomez-Pinilla, 2006; Rosas-Vargas *et al.,* 2011). The concentration of BDNF in the blood parallels that in the brain, where most circulating BDNF is produced (Forsgen *et al.,* 2011). Mutation in the BDNF gene leads to disturbed meal patterns and obesity (Fox & Byerly, 2004; Akkerman *et al.,* 2011), and reduced serum concentrations of BDNF are observed in obese people (El-Gharbawy *et al.,* 2006). BDNF is essential for body weight control, as suggested by low concentrations of circulating plasma BDNF in individuals with type 2 diabetes and obesity (Krabbe *et al.,* 2007) and the inverse relationship observed between BDNF concentrations and body mass index (BMI) in adults and children (Lommatzsch *et al.,* 2005). Furthermore, the deletion of the BDNF gene is associated with childhood obesity, decreased satiety and hyperphagia (Han *et al.,* 2008; Burns *et al.,* 2010). However, individuals suffering from type 2 diabetes can display decreased concentrations of plasma BDNF in the absence of obesity, suggesting that the regulating mechanisms of BDNF behind insulin resistance and obesity are different (Pedersen *et al.,* 2009).

### 5.1.4 Food Intake Regulation and BDNF

As reviewed in the General Introduction (Chapter 1, Section 1.1), food intake regulation is fundamentally involved in the maintenance of a stable body weight and energy homeostasis. This involves hormonal signaling in a range of brain regions, and BDNF is one of the key molecules currently being actively researched in this regard. Chronic i.c.v infusion of BDNF attenuates weight gain in rats by suppressing appetite (Lapchack *et al.,* 1992; Pelleymounter *et al.,* 1995; Gomez-Pinilla *et al.,* 2008), and this is thought to occur within the classic ‘satiety centre’, the VMH (Xu *et al.,* 2003). Repeated injections of BDNF administered directly to the VMH reduce body weight and insulin concentrations and reverse hyperleptinemia by decreasing feeding and increasing energy expenditure, physical activity and fat oxidation in DIO rodents (Godar *et al.,* 2011). These actions may be mediated by leptin, as central administration of leptin stimulates expression of the BDNF gene and its associated

protein in the VMH (Komori *et al*., 2006). However, the mechanisms behind this have yet to be fully determined. It is possible that the leptin interaction directly influences a signal transduction cascade which induces BDNF in the VMH (Rosas-Vargas *et al.,* 2011). BDNF is also responsive to diet composition. A diet high in SFAs has been shown to decrease its gene expression and circulating plasma concentrations (Stranahan *et al*., 2008), whereas, conversely, the intake of omega-3 PUFAs has been shown to increase them (Wu *et al*., 2004b; Vaynman & Gomez-Pinilla, 2006; Bousquet *et al.* 2009). Thus, in the current study, it was considered useful to determine whether the new high-PUFA feeding model could be defined by relationships between peripheral BDNF concentrations, indices of body weight, and meal patterns.

### 5.1.5 Research Question

***Does chronic consumption of a diet highly enriched in omega-3 fatty acids improve energy homeostasis in rat?***

### 5.1.6 Predictions

If so, then significant differences would be observed between morphometric, metabolic and behavioural indices of energy metabolism in rats fed diets highly enriched in omega-3 and saturated fatty acids derived from relatively pure sources and as compared to low-fat-fed controls.

### 5.1.7 Aims

To test these predictions by assessing diet-induced differences in

1. body weight and composition associated with
2. corresponding alterations in energy intake and meal patterns and
3. circulating concentrations of appetite-related factors.

### 5.1.8 Expected Outcomes

Rats fed the diet high in omega-3 fatty acids would display

1. attenuated weight gain
2. improved fat-to-lean ratio
3. reduced energy intake
4. changes in meal patterns, indicating enhanced satiety, and
5. reduced circulating concentrations of triglycerides and increased concentrations of BDNF. (Leptin, insulin and adiponectin could not be measured, so qualitative comparison with Chapter 3 would not be possible.)

## 5.2 Materials & Methods

### 5.2.1 Animals and Treatment

Age-matched adult male Wistar rats (~250 g) were stratified by body weight and randomly assigned to one of three groups (*n*=6/group). Two groups were fed isoenergetic diets for three weeks. These were enriched with either SFAs or PUFAs. Both diets provided 40% of energy from fat, mainly from coconut oil and a commercially purified source of the omega-3 fatty acid, docosahexaenoic acid (DHA; Incromega™ Marine Lipid Concentrate E1070, Croda Europe Ltd., Leek, Staffs, UK), respectively. These acted as controls for one another by eliminating the potentially confounding factor of differing energy intake. A third group, fed standard (low-fat) chow (providing 10% of energy from fat, from soybean oil) acted as a control for obesity induction, demonstrating normal weight gain and food intake. In addition to the changes in duration of exposure (due to time and financial constraints on the project) and in fatty acid sources and concentrations from those used in the original PUFA-fed characterisation study (Chapter 3), sucrose was also eliminated to avoid development of insulin resistance in control animals (see Table 5.1 for a summary of diet compositions and Appendix III & IV for full nutritional breakdowns).

As for the original study, group size was determined by Mead's resource equation (Chapter 2, Section 2.1.4). The larger, original group size (*n*=8) arose from initial requirements for only two treatment groups (high-SFA- and high-PUFA-fed). [In factorial design, sample size reduces as treatment groups increase (Festing *et al.,* 2004).] The third, control group was run separately several months later, when its need was realised and funds became available. All diets were manufactured by Research Diets, Inc. (NJ, USA). Body weight was measured weekly and food and water intake daily. Details of general husbandry and maintenance, as well a bioimpedance technique can be found in Chapter 2, Sections 2.1.6 and 2.5.2, respectively.

**Table 5.1. Nutritional composition of low-fat (control) and isoenergetic high-fat diets.**

| | Diet Type | | |
|---|---|---|---|
| **Macronutrient content** | **Control** | **SFA-enriched** | **PUFA-enriched** |
| Total energy content (kcal/gm) | 3.85 | 4.58 | 4.58 |
| % energy from carbohydrates | 70 | 40 | 40 |
| % energy from protein | 20 | 20 | 20 |
| % energy from fat | 10 | 40 | 40 |
| % energy from fat as SFAs | 30 | 95 | 1 |
| % energy from fat as PUFAs | 36 | 3 | 98 |
| % energy from fat as MUFAs | 34 | 2 | 1 |
| % energy from EPA & DHA (omega-3) | 8 | <0.05 | 78 |
| % energy from sucrose | 0 | 0 | 0 |

The high-PUFA diet and its high-fat control, enriched in SFAs, contained equal amounts of energy overall (isoenergetic) and contributed equal amounts of energy from fat. In contrast to those described in Chapter 3, fat sources were almost exclusively from PUFAs (specifically, omega-3 fatty acids) or SFAs in the respective diets. Note also that manufacture of specialised diets requires incorporation of a selection of carbohydrates, including sucrose (see Table 3.1, Chapter 3; Dr. L. Leiter, Research Diets, Inc., personal communication). However, due to the confounding influence of sucrose on insulin resistance and associated parameters, it was removed completely from the diets used here. **Abbreviations:** DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

### 5.2.2 Cell Proliferation Markers

Rats in the original characterisation study (Chapter 3) had received the cell proliferation tracer, BrdU, in their drinking water for the last week of dietary exposure. By the time the study described here was planned, the limitations of BrdU use were becoming clear. Thus, these animals did not receive BrdU, but were examined for other histological markers of cell proliferation instead (Chapter 7). As a potential stimulus for this process, a play tube was placed in each cage for the duration of the study for all groups.

### 5.2.3 Meal Pattern Data Acquisition and Analysis

Meal pattern information was gathered over a 24-hour period at the end of the study. Rats were removed from home cages and placed in specialised cages equipped with automated food intake monitoring systems, where they were given access to diets and water *ad libitum*. Raw data acquired in diurnal and nocturnal phases were analysed and are displayed according to previously published parameters: total

energy intake (kJ), number and duration (min) of meals, rate of feeding (g/min), inter meal interval (IMI; min) and satiety ratio (IMI/average energy content of the previous meal consumed; min/kJ; Cattone *et al.*, 2007; Farley *et al.*, 2003; Hariri & Thibault, 2011). Details of the cage and software analysis system can be found in Chapter 2, Section 2.4.3.

### 5.2.4 Blood Chemistry

After an overnight fast, and under brief gaseous anaesthesia, blood samples were collected from the tail vein at baseline and weekly thereafter and plasma separated by centrifugation. Blood glucose concentrations were measured immediately by glucose-oxidase strips and a hand-held glucose meter. Plasma concentrations of triglycerides (TGs) were measured by (enzymatic, colorimetric) diagnostic kit and concentrations of BDNF by ELISA, according to manufacturers' protocols. Details of suppliers and manufacturers' protocols can be found in Chapter 2, location 2.6.2. Plasma concentrations of leptin, adiponectin and insulin, measured in the original characterisation study (Chapter 3) were not measured here, due to financial constraints.

### 5.2.5 Body Composition Analysis & Termination

Under deep anaesthesia, final whole-body composition was assessed by bioimpedance (EM-SCAN/TOBEC® SA-3114 Small Animal Body Composition Analysis System, Royem Scientific, Luton, UK). Rats were then perfuse-fixed and brains removed for histological analyses described in Chapter 7. The descending aorta was clamped to prevent fixation of peripheral tissues required fresh for this study. White adipose tissue (WAT) pads, interscapular brown adipose tissue (BAT), gastrocnemius and soleus muscle and liver were dissected free, weighed, snap-frozen in liquid nitrogen, and stored at -80°C for further analysis. Tissue weights were expressed as a percentage of final body weight.

### 5.2.6 Statistical Analysis

Data are expressed as mean ± SEM. All tissue masses are presented as a relative percentage of final body weight. Differences between diet groups in three-way comparisons were determined using one-way ANOVA with post-hoc Bonferroni correction and considered significant at critical $p<0.0166$ for 3 comparisons.

Differences between diet groups over a time course were determined using 2-way ANOVA with repeated measures and post-hoc Bonferroni correction and considered significant at $p<0.05$. In all instances comparisons demonstrated roughly equal variation and therefore the sphericity criteria were met and consequently variation data is not presented here. Statistical analysis was carried out using software SPSS *v*19. Justification for use of all statistical analyses can be found in Chapter 2 section 2.11.

## 5.3 Results

### 5.3.1 Energy Intake & Body Weight

PUFA (omega-3)- and SFA-fed rats consumed comparable amounts of energy over the course of the study ($p>0.05$), which were greater than that consumed by controls (+13±1.1% in SFA-fed animals, +12±1.8% in PUFA-fed animals, both $p<0.01$; Figs. 5.1A & B). This, and other significant effects presented here, are similar to those described in Chapter 3, but after less than half the duration of dietary exposure (3 *vs.* 8 weeks). Increased energy intake in the SFA-fed group did not induce weight gain (Figs. 5.2A-C). Despite an equivalent energy intake, body weight was attenuated in the PUFA-fed rats upon termination (-9±0.9% and –11±1.3% *vs.* control and SFA-fed, respectively; $p<0.05$; Fig. 5.2A). Body weight gain was also attenuated in PUFA-fed animals (-31±2.8% and –38±4.3% *vs.* control and SFA-fed, respectively; both $p<0.01$; Figs. 5.2B & C). Energy efficiency was predictably reduced in this group (-28±4.5% *vs.* control, $p<0.01$; Fig. 5.2D).

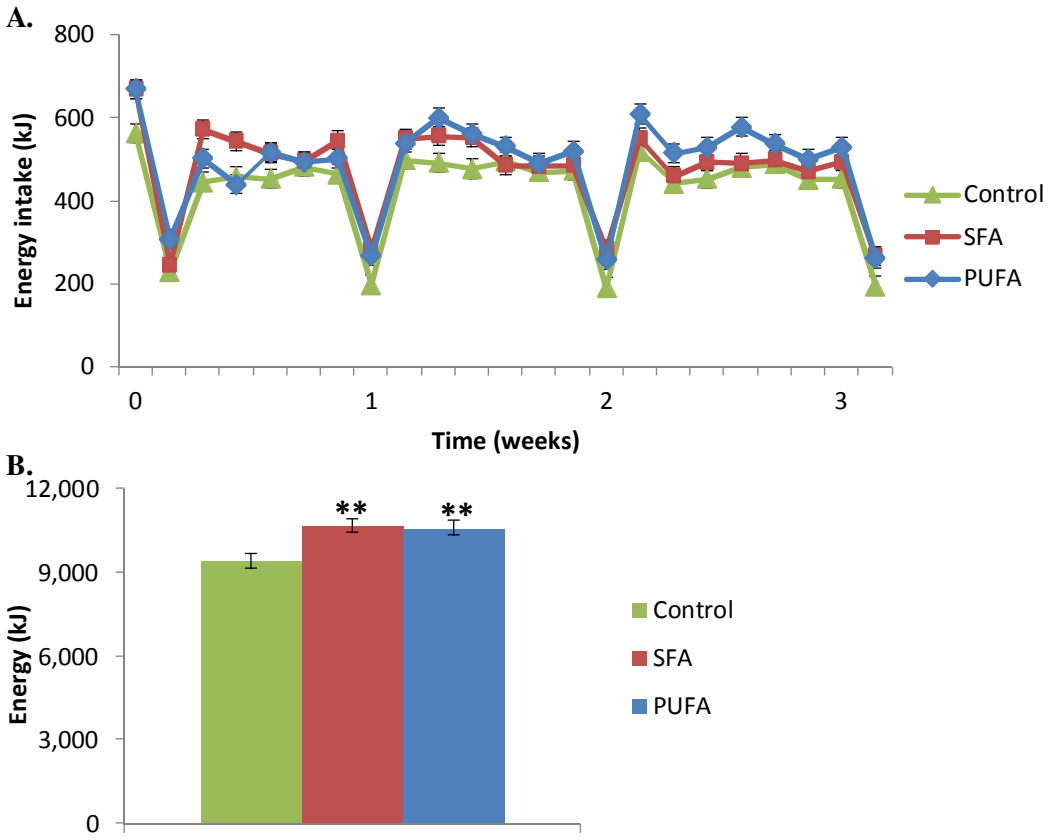


**Figure 5.1.** Energy intake evolution (A) and cumulative energy intake (B) in control, SFA- and PUFA (omega-3)-fed rats consuming diets for three weeks. Reduced intake at weekly intervals (A) reflects imposed overnight fasts prior to blood sampling. **Overall energy intake was increased equally in high-fat-fed groups.** Values are expressed as mean ± SEM ($n$=6/group): **$p<0.01$ compared to controls (1-way ANOVA).

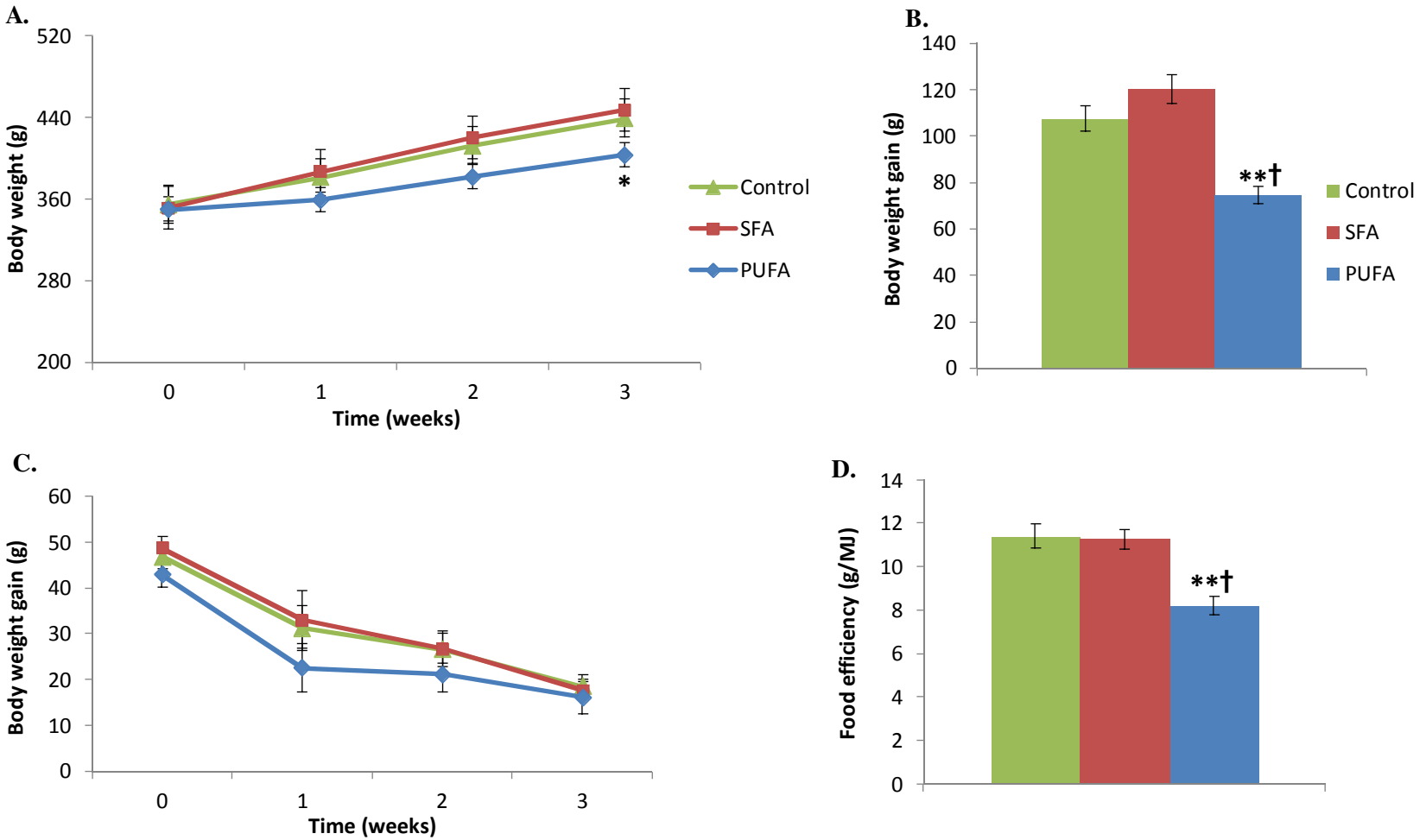


**Figure 5.2.** Absolute body weight evolution (A), cumulative weight gain (B), weight gain evolution (C) and food efficiency (D) in control, SFA- and PUFA (omega-3)-fed rats consuming diets for three weeks. **High-omega-3 feeding attenuated weight gain, reflected in reduced food efficiency.** Values are expressed as mean ± SEM ($n$=6/group): *$p$<0.05 compared to controls (2-way ANOVA with repeated measures); **$p$<0.01 compared to controls; † compared to SFA (1-way ANOVA). Food efficiency = Δbody weight (g)/Σfood intake (MJ).

### 5.3.2 Terminal Tissue Mass and Body Composition

High-SFA feeding had no effect on adiposity, despite the associated increase in energy intake, but consistent with unchanged body weight gain. Attenuated weight gain in PUFA (omega-3)-fed rats was reflected in smaller fat pad masses across all WAT depots examined, significantly so in the epididymal and perirenal depots (-37±4.5% and -35±2.8%, and –26±3.7% and -28±4.1%, respectively, *vs.* control and SFA-fed; $p<0.01$; Fig. 5.3A), and in dramatic contrast to induction of adiposity in this group in Chapter 3. Despite the suggestion, by these combined results, of raised energy expenditure in the PUFA-fed group, BAT mass was unaffected (Fig. 5.3B). Muscle mass was unaffected by diet (Fig. 5.3D); thus, reduced adiposity by high-PUFA feeding was reflected in improved body composition (fat-to-lean ratio; -36±3.8% *vs.* control and -32±2.6% *vs*. SFA-fed; both $p<0.01$; Fig. 5.3E). There was no effect of diet on liver mass (Fig. 5.3C).

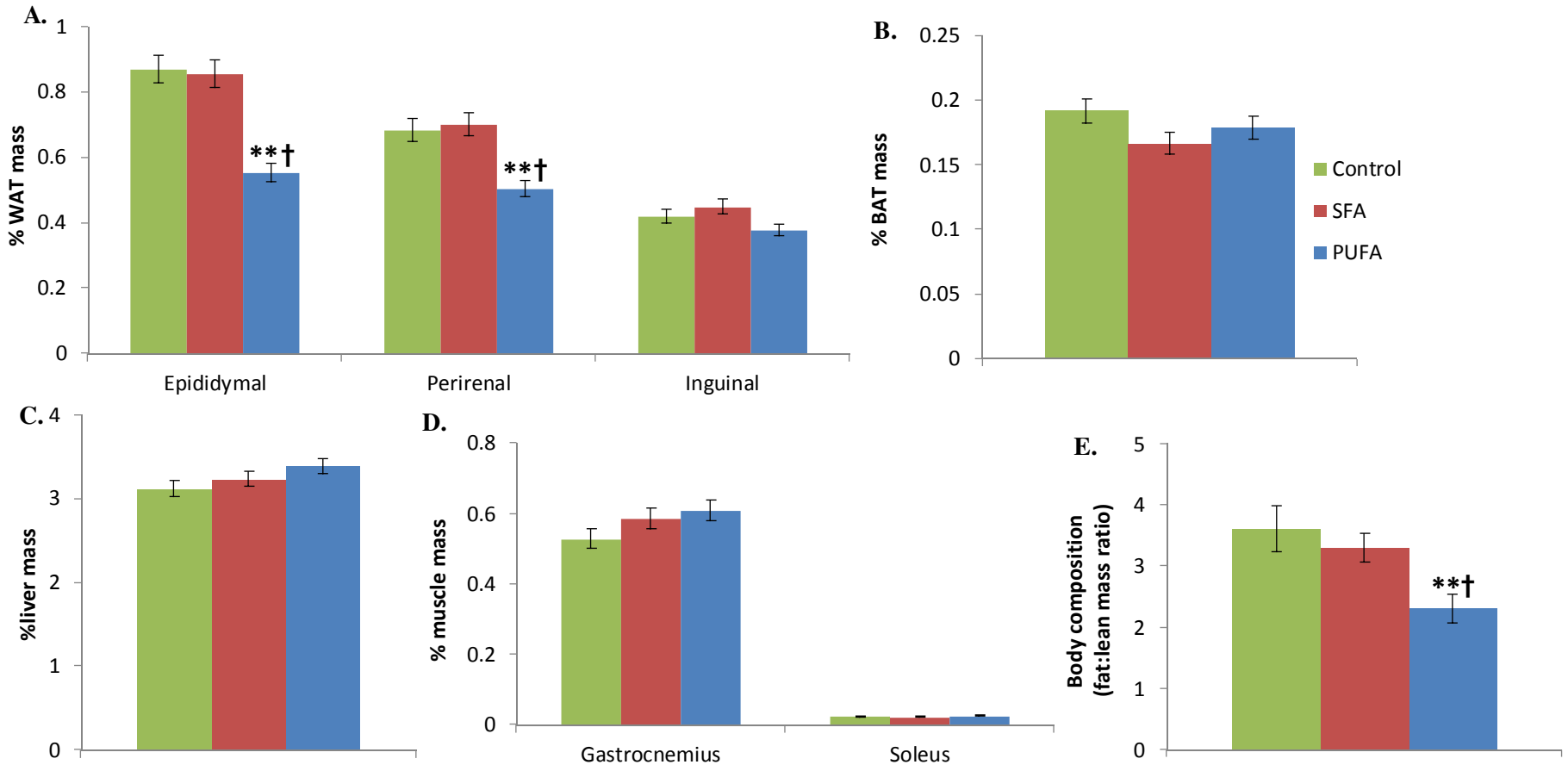


**Figure 5.3.** Tissue masses relative to final body weight in control, SFA- and PUFA (omega-3)-fed rats after consumption of diets for three weeks: WAT (A), BAT (B), liver (C) and muscle (D). Body composition (E) was calculated as the ratio of summed WAT depot to muscle masses. **High-omega-3 feeding reduced adiposity, reflected in enhanced body composition.** Values are displayed as mean ± SEM ($n$=6/group): **$p$<0.01 compared to controls; †$p$<0.01 compared to SFA (1-way ANOVA).

### 5.3.3 Bioimpedance Analysis

Terminal bioimpedance analysis (Table 5.2) showed that assessment of whole-body composition was in general agreement with that determined by surrogate measures (select tissue masses; Figs. 5.3A-E). None of the measures were affected by high-SFA feeding, but high-PUFA (omega-3) feeding reduced total fat and lean mass compared to control and SFA-fed groups (both $p<0.01$). The reduction in overall lean mass, in addition to fat mass, suggests a reduction in whole-body weight, consistent with findings (Figs. 5.2A-C), and may explain why the relative total fat mass (%) was unchanged by high-PUFA feeding. The discrepancies with surrogate findings may result from the fact that the discrete tissues selected did not reflect whole-body change; i.e. other fat depots and skeletal muscles which were not examined may have responded to the diets differently.

**Table 5.2. Diet effects on whole-body composition.**

| | **Control** | **SFA** | **PUFA (omega-3)** |
|---|---|---|---|
| **Lean body mass (g)** | 198±6.3 | 203±5.9 | 184±3.4**† |
| **Total body fat (g)** | 241±6.4 | 243±4.7 | 219±3.1**† |
| **Total body fat (%)** | 55±0.5 | 54±0.3 | 53±0.8 |
| **Fat-free mass (g)** | 69±2.6 | 72±2.3 | 66.8±1.5 |

**Terminal bioimpedance scores show that three weeks of high-omega-3 feeding reduce overall mass.** Values are displayed as mean ± SEM ($n$=6/group): **$p<0.01$ compared to controls; †$p<0.01$ compared to SFA (1-way ANOVA).

### 5.3.4 Circulating Factors

#### 5.3.4.1 Whole-Blood Glucose Concentrations

Analysis by two-way ANOVA with repeated measures demonstrated no differences in whole-blood glucose concentrations between diet groups ($F(2, 15) = 0.2085$, $p=0.8141$), over time ($F(3, 45) = 2.076$, $p=0.1168$) or any interaction between diet and time ($F(6, 45) = 0.7208$, $p=0.6349$). Similar to findings presented in Chapter 3, there were no differences in whole-blood glucose concentrations between diet groups over the course of the study (all $p>0.05$), and within-group, terminal concentrations had changed very little from baseline: -2±0.1%, +20±2.2% and +16±2.8% in control, SFA- and PUFA-fed groups, respectively (Fig. 5.4).

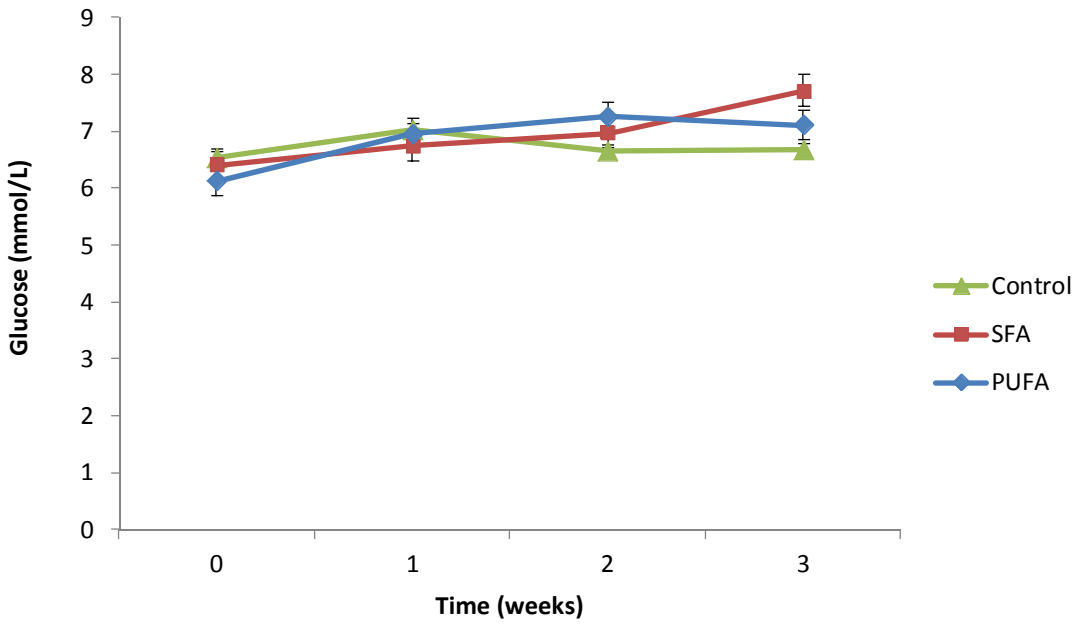


**Figure 5.4.** Fasting whole-blood glucose concentrations in control, SFA- and PUFA (omega-3)-fed rats consuming diets for three weeks. **These were unaffected by high-fat feeding.** Values are expressed as mean ± SEM ($n=6$/group): all $p>0.05$ (2-way ANOVA with repeated measures).

### 5.3.4.2 Plasma BDNF Concentrations

Analysis by two-way ANOVA with repeated measures demonstrated no differences in circulating BDNF concentrations between diet groups (F (2, 15) = 1.232, $p$=0.3196), over time (F (3, 45) = 1.842, $p$=0.1530) or any interaction between diet and time (F (6, 45) = 0.5037, $p$=0.8022). There were no differences in circulating BDNF concentrations between diet groups over the course of the study (all $p$>0.05). All three dietary groups show an increase in circulating concentrations by the end of the study with respect to baseline (+189±18.9%, +76±11.2% and +42±5.4% in PUFA (omega-3), control and SFA-fed animals, respectively; Fig. 5.5).

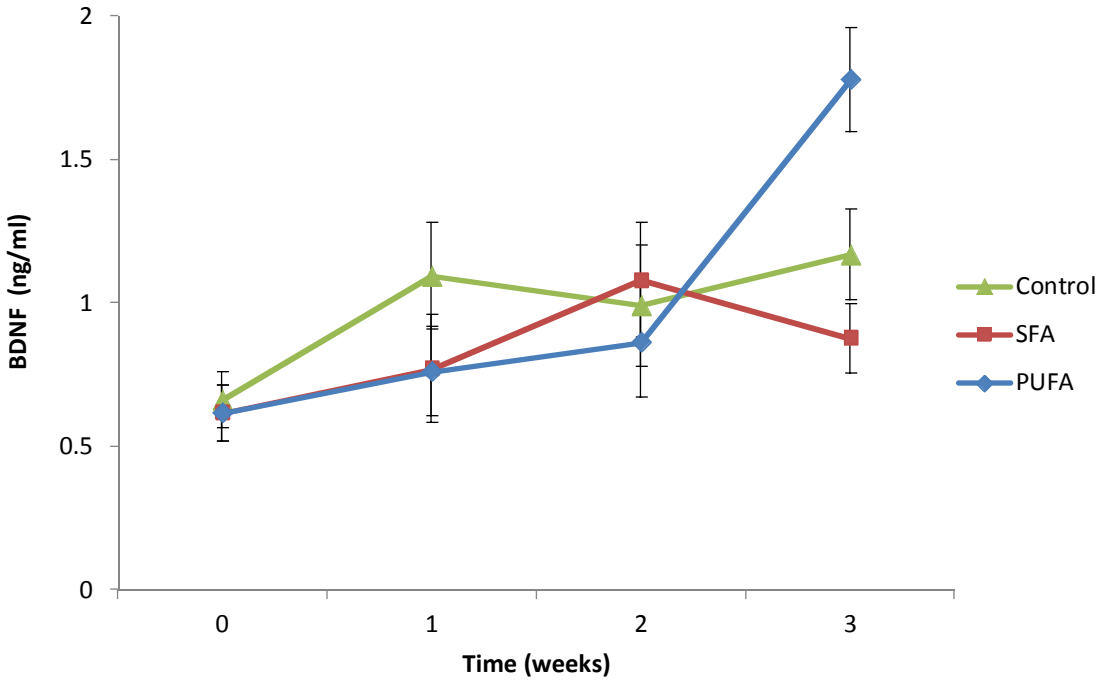


**Figure 5.5.** Plasma BDNF concentrations in control, SFA- and PUFA (omega-3)-fed rats consuming diets for three weeks. **These were not significantly affected by high-fat feeding.** Values are expressed as mean ± SEM ($n$=6/group): all $p$>0.05 (2-way ANOVA with repeated measures).

### 5.3.4.3 Plasma Triglyceride Concentrations

Analysis by two-way ANOVA with repeated measures demonstrated a significant difference in circulating triglyceride concentrations between diet groups ($F(2, 15) = 5.271$, $p=0.0185$) and over time ($F(3, 45) = 5.853$, $p=0.0018$), but no interaction between diet and time ($F(6, 45) = 1.667$, $p<0.1513$). Similar to results in Chapter 3, though the expected relationship between high SFA consumption and an increase in circulating TG concentrations was again not observed, a dramatic and progressive fall was observed in the PUFA (omega-3)-fed animals (-60±5.4% from baseline; -65±6.8% and -68±4.7% *vs.* control and SFA-fed respectively; both $p<0.05$) (Fig. 5.6), a reduction 16±1.8% greater than that seen in Chapter 3 (as confirmed by AUC analysis).

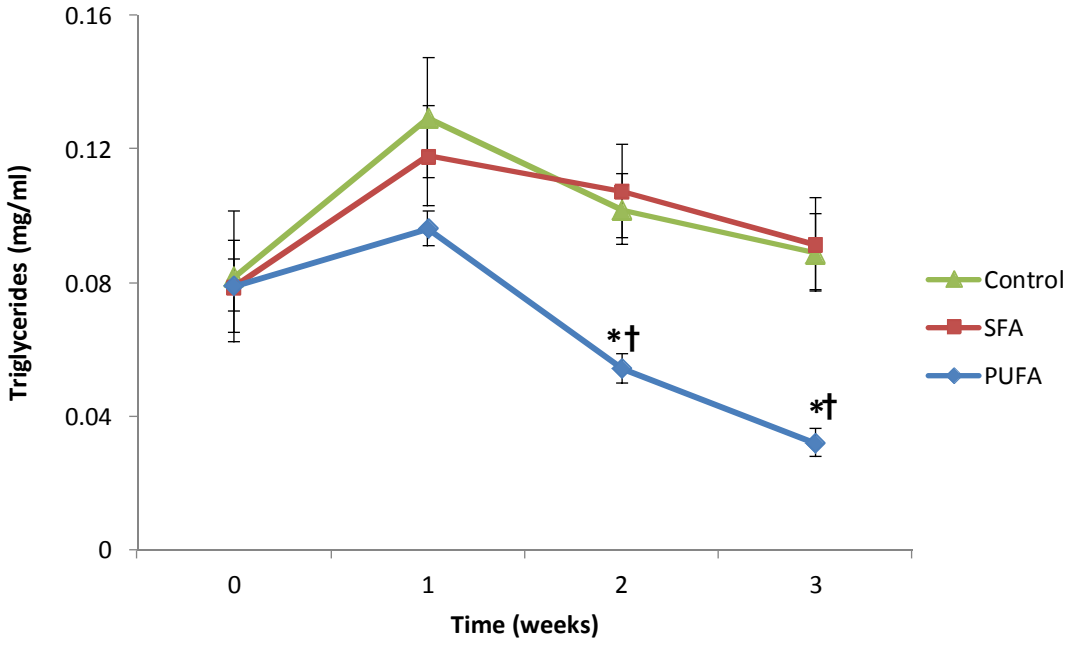


**Figure 5.6.** Plasma triglyceride concentrations in control, SFA- and PUFA (omega-3)-fed rats consuming diets for three weeks. **High-omega-3 feeding progressively reduced triglyceride concentrations from week 1.** Values are expressed as mean ± SEM ($n$=6/group); *$p<0.05$ compared to controls; †$p<0.05$ PUFA compared to SFA (2-way ANOVA with repeated measures).

### 5.3.5 Meal Patterns

Similar to results presented in Chapter 3, diet appeared to have no significant effect on diurnal or nocturnal energy intake, when analysed separately after three weeks of consumption (all $p>0.01$; Fig. 5.7A). The overall lack of effect conflicts with the increase in cumulative 24-hour intake observed here in both SFA- and PUFA (omega-3)-fed groups (Fig. 5.1B), but could reflect the fact that other meal pattern changes offset one another; i.e. increased diurnal, but decreased nocturnal, meal duration induced by SFA feeding (+26±1.6% and -21±2.9% *vs.* controls, respectively; both $p<0.01$; Figs. 5.7C) and nocturnal reduction in number of meals, but also feeding rate, induced by PUFA feeding (-33±2.4% and -23±1.8% *vs.* controls, respectively; both $p<0.01$; Figs. 5.7B&D).

Increased diurnal meal duration in SFA-fed animals was reflected in decreased intermeal interval (IMI) and satiety during the day (-34±3.8% and -48±2.7% *vs.* controls, respectively; Figs. 5.8A, C). Though reduced nocturnal meal duration in this group might predict extended IMI and enhanced satiety at night, nocturnal satiety measures were unchanged, possibly because the slight (albeit non-significant) increase in meal numbers was compensated by the slight reduction in feeding rate at night (Figs. 5.8B, D). Similarly, although PUFA-fed animals took fewer meals at night (Fig. 5.7B), they also slowed their rate of feeding (Fig. 5.7D), consistent with unchanged satiety overall (Figs. 5.8B, D).

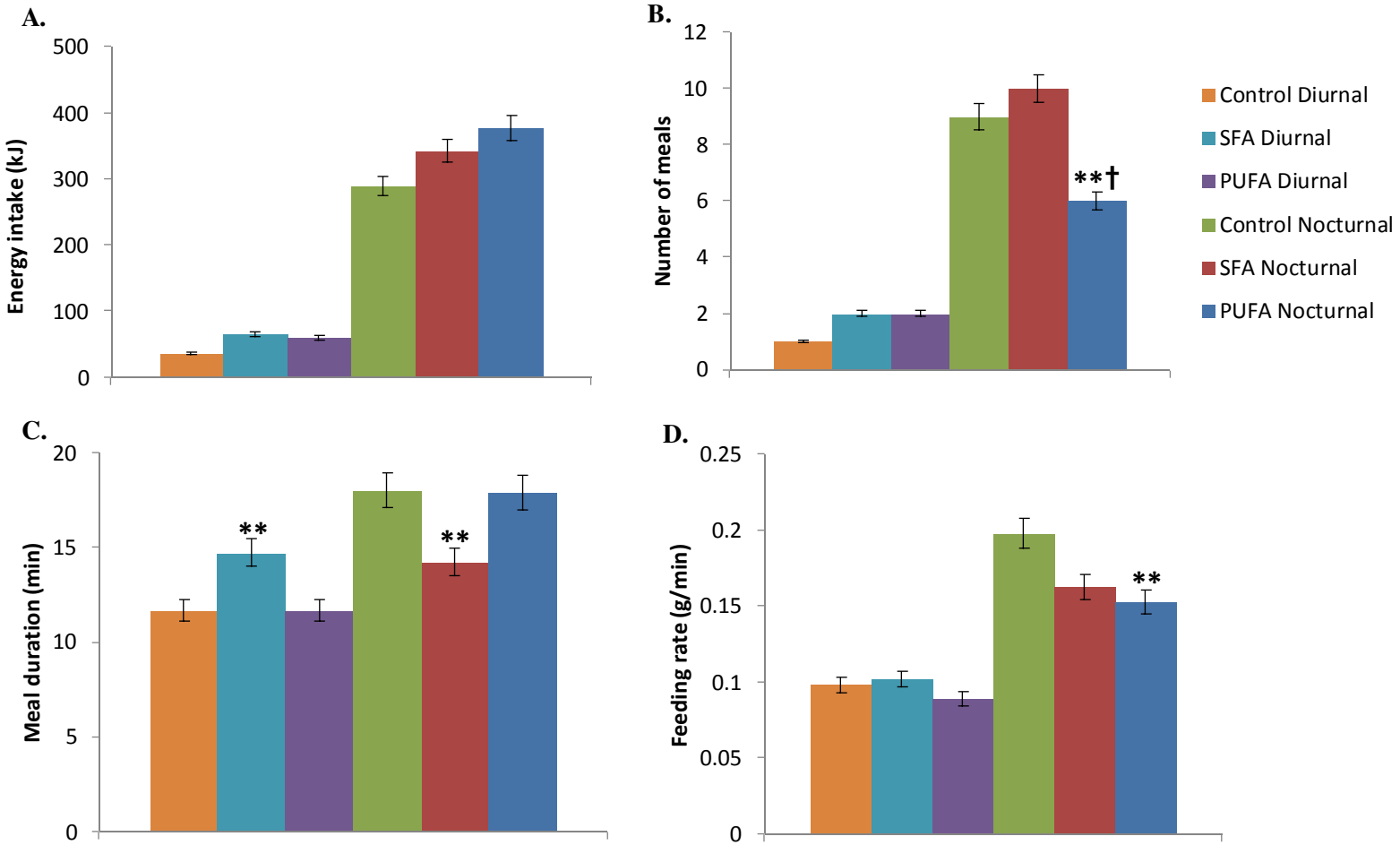


**Figure 5.7.** Final 24-hour diurnal and nocturnal energy intake (A), number of meals (B), meal duration (C) and feeding rate (D) in control and high-SFA- and -PUFA (omega-3)-fed rats consuming diets for three weeks. **High-omega-3 feeding altered nocturnal meal patterns by reducing the number of meals taken and rate of feeding. In contrast, high-SFA feeding altered meal duration during both phases.** Values are expressed as mean ± SEM ($n$=4/group): **$p$<0.01 compared to controls; †$p$<0.01 compared to SFA (1-way ANOVA).

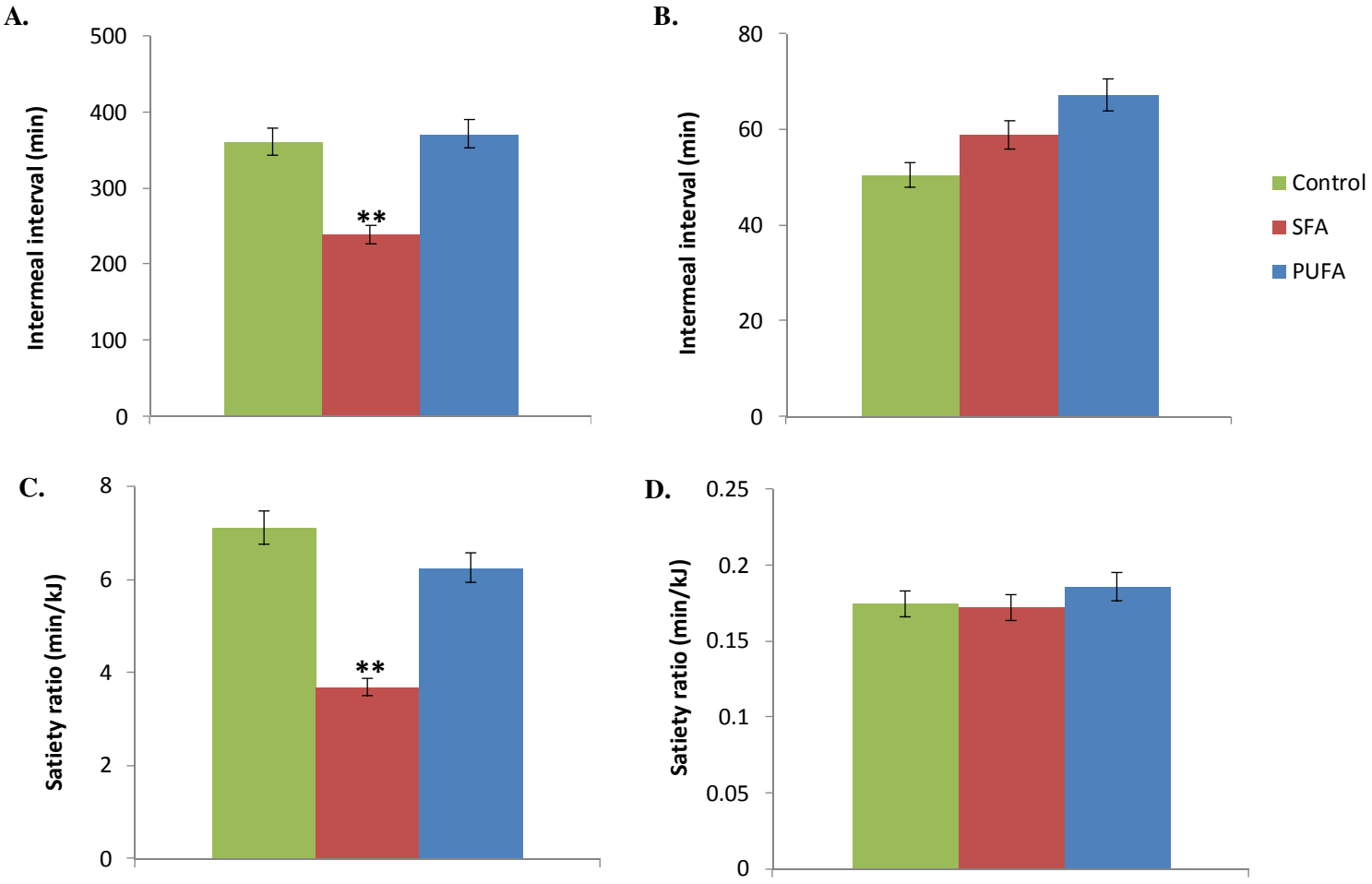


**Figure 5.8.** Final 24-hour diurnal and nocturnal intermeal interval (A, B), and satiety ratio (C, D) in control, SFA- and PUFA (omega-3-fed)-fed rats consuming diets for three weeks. **High-SFA feeding reduced daytime satiety, but high-PUFA feeding had no effect during either phase.** Values are expressed as mean ± SEM ($n$=4/group); **$p$<0.01 compared to controls (1-way ANOVA).

### 5.3.6 Circadian Rhythmicity

Feeding rhythmicity data gathered over the final 24 hours of this three-week study show clearly that in both SFA- and PUFA (omega-3)-fed groups, energy intake per meal generally exceeded that of controls (Fig. 5.9). This is consistent with summary measures of energy intake (Fig. 5.1B & 5.7A). All groups consumed the majority of their meals at night, as expected in a nocturnal species, and these were more intermittent in the experimental groups than the controls (Table 5.3). Furthermore, these meals appeared to be taken in small clusters of two or three in PUFA-fed animals, whereas those of the other two groups were more evenly spread across the phase. Both control and SFA-fed animals consumed two meals just prior to 'lights off', the second meal being larger than the first in both cases, but markedly so in the case of the SFA-fed group. In contrast, the extent of diurnal feeding in the PUFA-fed group was the consumption of a single meal around midday. Nocturnal meals began at 'lights off', whereas feeding activity was absent from this period in the SFA-fed group. Finally, comparison of these patterns to those presented for week 3 in Chapter 3 show a marked reduction here, by almost half, in the number meals taken at night in both SFA- and PUFA-fed animals, with less consistent meal sizes (i.e. a greater range in energy intake per meal) in the latter compared to their PUFA-fed counterparts in Chapter 3.

**Table 5.3. Ranges of values associated with the circadian rhythmicity of feeding.**

| ***Diurnal*** | ***Week 3*** | | |
|---|---|---|---|
| **Range** | **Control** | **SFA** | **PUFA** |
| **Consumption window** | 18.30-20.00 (1.5 hours) | 19.00-20.00 (1 hour) | 12.30-13.00 (0.5 hours) |
| **Number of meals** | 2 | 2 | 1 |
| **Energy intake/range of meal (kJ)** | 4-14kJ (10kJ) | 6-43kJ (37kJ) | 3kJ |
| ***Nocturnal*** | ***Week 3*** | | |
| **Range** | **Control** | **SFA** | **PUFA** |
| **Consumption window** | 21.00-06.30 (9.5 hours) | 22.00-07.30 (9.5 hours) | 20.00-07.30 (11.5 hours) |
| **Number of meals** | 12 | 8 | 10 |
| **Energy intake/range of meal (kJ)** | 2-30kJ (28kJ) | 8-51kJ (43kJ) | 11-60kJ (49kJ) |

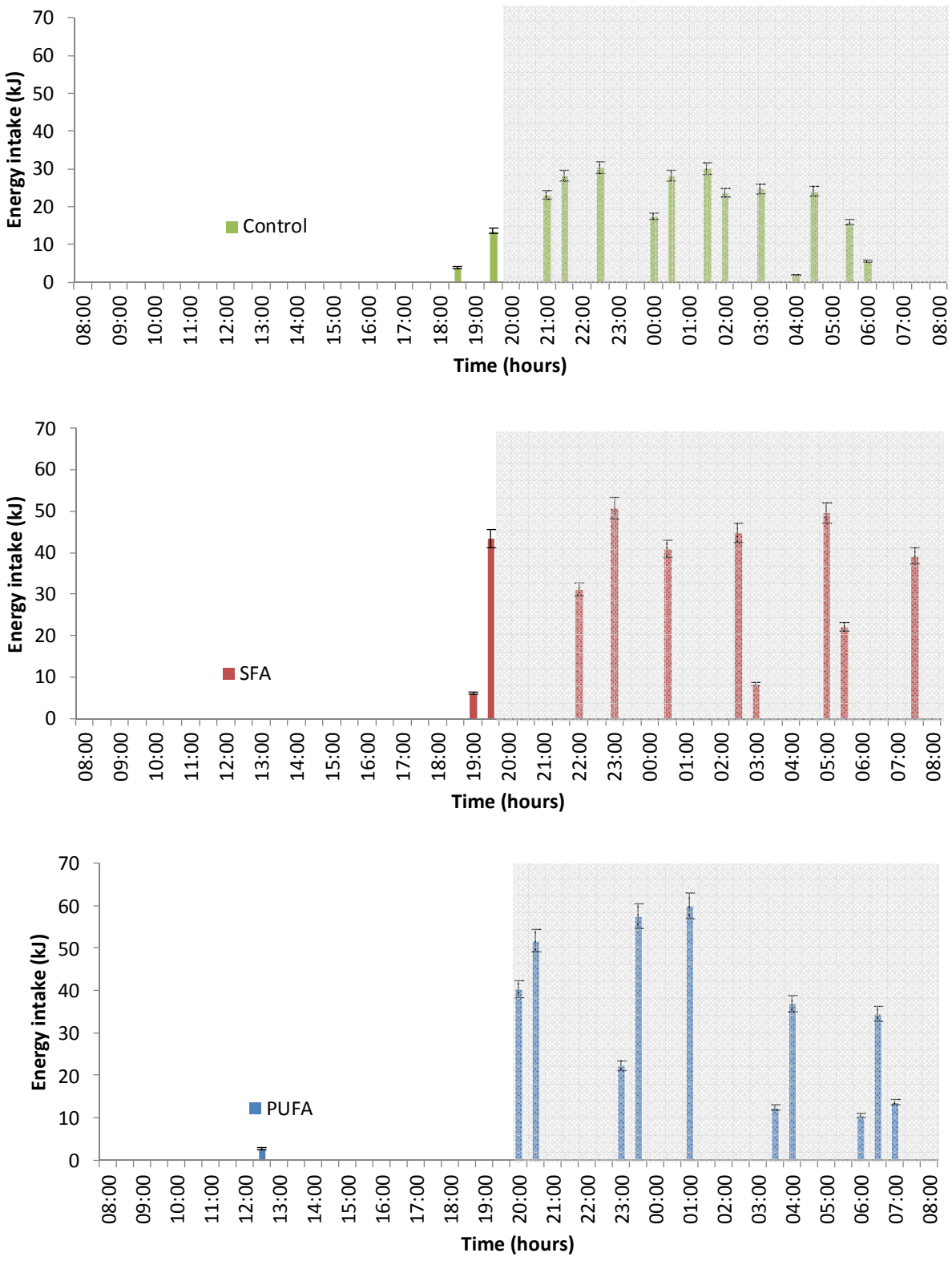


**Figure 5.9.** Circadian rhythmicity of feeding activity after three weeks of control, SFA- and PUFA (omega-3) diet consumption. Data are presented as means of 30-minute intervals over the final 24 hours of feeding. Values are expressed as mean ± SEM ($n$=4/group). Grey shading represents the nocturnal phase.

## 5.4 Discussion

### 5.4.1 Morphological and Metabolic Phenotype

Overall, the refined high-PUFA (omega-3) feeding model appeared to be a valid one, when compared to published findings in humans and animals. Over the course of only three weeks, PUFA-fed rats displayed significant improvements in key measures of body weight homeostasis, in comparison to both high-SFA- and low-fat-fed control groups. However, the lack of separation between these two groups on most parameters calls into question the suitability of coconut oil as the SFA source in a high-fat control diet, at least under shorter-term exposure conditions.

Although this study cannot be directly compared with the original study (Chapter 3), their apparent similarities and differences are worth mentioning. Here, in contrast to the original high-PUFA-fed group, high-omega-3 feeding attenuated body weight gain by reducing adiposity. This was in spite of increased energy intake, comparable with that consumed by the high-SFA-fed animals. This suggests that the omega-3 FAs enhanced energy expenditure, which is a known outcome of their consumption (Buckley & Howe, 2009). Though not reflected in a change in BAT mass, it may have been revealed in molecular and histological measures of increased thermogenesis; for instance, mice fed a diet enriched in omega-3 FAs for four weeks have shown increased BAT thermogenic activity, as measured by increased GDP binding, BAT hyperplasia and mitochondrial content (Oudart *et al.,* 1997).

Alternatively, increased expenditure may have been detectable by calorimetry or measures of increased locomotor activity (Kemp, 1999). As in the original study (Chapter 3), diet had no effect on skeletal muscle mass, such that the surrogate body composition ratio was, in fact, a measure of adiposity. Reduced adipose mass in the omega-3-fed group was reflected in reduced food efficiency, suggesting that omega-3 FAs were being utilized to satisfy immediate energy demands, rather than going into storage (Buckley & Howe, 2009; Puglisi *et al.,* 2011), which, again, could have been supported by observation of increased physical activity. Indeed, PUFAs are oxidised more rapidly, decreasing available time for deposition (DeLany *et al*., 2000; Bell & O'Keefe, 2011). This occurs as PUFAs influence the up-regulation and expression of genes involved in FA oxidation in organs such as the liver and

intestine, and the down-regulation of genes in adipose tissue that promote lipogenesis (Buckley & Howe, 2009).

Bioimpedance analysis expanded on these findings to reveal that attenuation of weight gain was due to a combined whole-body reduction in fat and lean mass (see Results, Section 5.3.3), such that relative fat mass was unchanged by high-omega-3 feeding. Whether this represents an attenuation of overall growth would require a longer duration study. As lean mass is now considered to be an important peripheral driver of energy metabolism (Blundell *et al.,* 2012), its overall reduction could represent a detrimental effect of such a high concentration of an omega-3 FA in the diet, compromising normal function. Although findings are inconsistent, in general, omega-3 FAs appear to stimulate nutrient delivery to skeletal muscles and alter gene expression involved in metabolism to increase lean mass (Demmig-Adams & Carter, 2007). Once again, the short duration of the current study could explain this discrepancy.

Studies have shown that composition of dietary FAs affects whole-body distribution of adipose tissue differentially (Hill *et al*., 1992; 1993). This is consistent with the differential effects of the omega-3 diet on the three fat depots examined. Considered together with the corresponding results in Chapter 3, these results are consistent with previous findings that increased WAT mass is observed in rats fed diets high in SFAs, compared to those fed diets high in omega-3 PUFAs (Parrish *et al*., 1990; Hill *et al*., 1992; Belzung *et al*., 1993; Hainault *et al*., 1993; Hill *et al.,* 1993; Flachs *et al*., 2006; Okere *et al*., 2006; Fernandez-Quintela *et al*., 2007). Numerous studies have shown that incorporating omega-3 PUFAs, including DHA, into high-fat diets reduces body fat accumulation in rodents (Cunnane *et al.,* 1986; Belzung *et al*., 1993; Hainault *et al.,* 1993; Baillie *et al.,* 1999; Ruzickova *et al.,* 2004). This appears to occur after both long- and short-term exposure. Reduced body weight has been observed in mice fed diets supplemented with 10% cod liver oil for several months, in the absence of any change in energy intake (Cunnane *et al*., 1986). However, a similar effect has been observed in rats fed for only 16 days on a diet high in fat (50% of energy provided as fat), where 15% of the fat was sourced from fish oil. They showed 30% reductions in subcutaneous and visceral fat mass, despite food intake and body weight similar to that of a low-fat-fed control group (10% of energy provided as fat) (Hainault *et al*., 1993).

This protective effect of PUFAs against fat accumulation is now well-recognised and is thought to be concentration-dependent. Of rats fed diets containing high (40.6% of energy), medium (18.6% of energy) and low (0.7% of energy) omega-3 FA content for one month, those receiving the highest concentration displayed the greatest reduction in epididymal and retroperitoneal fat accumulation. This was explained by the lowering of circulating TGs, which was also concentration-dependent (Belzung *et al.* 1993). Although increasing the omega-3 FA concentration to 78% from 25% in the original study did not result in any greater reduction in TG levels here (both reaching a minimum of 0.04 mg/ml), it appears that this latter reduction may have been continuing on a downward trajectory (Fig. 5.7), rather than cycling around a plateau (Fig. 3.10). The mechanisms behind reduction of circulating TG levels by PUFAs are explained in Chapter 3 (Section 3.4.5).

Furthermore, the observed reduction in visceral fat, of which peritoneal and epididymal are constituent, is thought to be mediated primarily by DHA, as opposed to EPA. This has been demonstrated in mice fed high-fat diets containing mixtures of the two omega-3 FAs for five weeks. It was found that DHA, rather than EPA, attenuated the accumulation of epididymal fat, but neither had an effect on the accumulation of subcutaneous fat, suggesting that DHA, rather than EPA, was primarily responsible for promoting the anti-obesity effect (Ruzickova *et al.,* 2004). This is consistent with the unchanged inguinal (subcutaneous) mass seen here, and highlights the importance of considering relative concentrations of the two omega-3 FAs when making dietary recommendations.

Finally, PUFA consumption has previously been associated with augmented liver size, and specifically, DHA is readily incorporated into liver phospholipids, increasing the overall mass of the organ (Hill *et al*., 1993; Rapoport *et al*., 2010). However, there was no effect of diet on liver mass in the current study, suggesting that three weeks of consumption was not a sufficient length of time for this effect to manifest. This is further supported by the findings in Chapter 3 that eight weeks of consumption was sufficient to increase liver mass in the PUFA-fed group, above that of both the control and SFA-fed group.

### 5.4.2 Plasma BDNF Concentrations

There was no effect of diet on circulating concentrations of BDNF. Had dietary exposure been extended, then rats fed the PUFA diet may have demonstrated an increase in BDNF concentrations, as an elevation in BDNF secretion in rats has been observed after one and ten months' PUFA consumption (Wu *et al.,* 2004b; Bousquet *et al.* 2009). A reduction in BDNF levels in the high-SFA-fed group may also have become apparent as SFA consumption has been shown to reduce concentrations in rats fed for two and eight months (Molteni *et al.,* 2002; Stranahan *et al*., 2008). Thus, an extended dietary exposure may be required to demonstrate the relationships between plasma BDNF concentrations and body weight described in the Introduction (Section 5.1.3) before an affect of FA type can be determined.

### 5.4.3 Meal Patterns & Circadian Rhythmicity

Consistent with original findings in Chapter 3, the high-fat diets here did not disrupt the overall temporal sequence of feeding (diurnal-nocturnal partitioning). With all diets, diurnal meals were again consistently smaller and less frequent than nocturnal ones, as expected for the active phase of the rat (Strubbe & Woods, 2004). Also similar was the failure of energy intake, as determined by meal pattern analysis, to fully reflect the cumulative, crudely measured increase in intake observed over the course of the study (Figs. 5.1A&B). Increases were, in fact, observed during both the light and dark phases, in both studies, but were minor and non-significant. This could be due to the fact that the automated cages represented a new environment, for which rats required a longer acclimatization period than 24 hours in order for normal responses to the diets to fully emerge. However, statistical comparison of energy intake between days 1 and 3 of the 72-hour meal analysis period in the original study did not reveal any differences in any group (data not shown), suggesting that acclimatization might have to be extended beyond three days.

Meal duration was unchanged by diet in the original study, but here was disrupted in the high-SFA-fed group. Longer meals taken in the day were offset by shorter meals at night. Statistically unchanged glucose concentrations in this group suggest that the usual relationship between glucose and meal-to-meal control of feeding behaviour is lost on this diet. However, the slight, non-significant rise in

glucose levels in this group at the end of the study, suggesting increased hunger (Bray, 2000), does correlate with the reduced diurnal satiety observed in this group.

Meal pattern signature in high-omega-3-fed animals was defined by changes on different parameters (decreased number of meals and feeding rate), and only at night, but which would also offset one another. These suggest enhanced nocturnal satiety, in this group, which was, in fact, observed, though not to a significant extent. Had a significant difference been drawn out, for instance, by a longer duration of exposure to the diet, it would have contrasted with the PUFA-induced satiety occurring in the opposite phase (daytime) in the original study, and suggested a differential effect on temporal sequence by individual omega-3 FAs and/or their concentrations; i.e. in the current study, omega-3 FAs provided three times the energy from fat as those in Chapter 3, as well as being comprised of a higher DHA:EPA ratio (126:17 *vs.* 19:22; see Appendices II and IV).

Despite a general absence of obese phenotype in the SFA-fed group on morphometric and metabolic parameters (unchanged adiposity and TG levels), the characteristic feeding pattern associated with obesity, of fewer and larger meals, was observed during the nocturnal phase (Fig. 5.9 and Table 5.3). This appears to conflict with the subtle increase in number of meals shown in the summary analysis (Fig. 5.7B) because the circadian rhythmicity graphs show average meal numbers in a 30 minute interval, a disadvantage of the binning technique. However, this was also apparent in the omega-3-fed group, which had shown marked improvements on these same phenotypic measures. Thus, the influence of FAs on energy homeostasis may not be expressed at all levels of feeding behaviour. Rhythmicity of feeding in response to individual FAs may vary according to their type. Although here it appears to be independent of FA type, in other studies, it does not (Hariri & Thibault, 2010). As these findings are being reported in relation to a pure omega-3 source for the first time, more studies are needed to determine if this independence is, indeed, typical of this family of FAs. On the other hand, three features of nocturnal periodicity appeared to differ between the SFA- and omega-3-fed groups. These were energy intake per meal, which showed the greater variability in the latter group, the pairing of lower- with higher- energy meals, also in the latter group, and the timing of the first nocturnal meals immediately after ‘lights off’ (the very start of the dark phase) in this group, compared with its possible counterpart just before ‘lights

off' in the SFA-fed group (the very end of the light phase). This suggests fulfillment of immediate energy and lipogenesis requirements (Armstrong *et al.,* 1978) at times which are slightly offset in the two groups, possibly indicating effects of the different FAs on the central clock (Mendoza *et al.,* 2008).

## 5.5 Conclusion

In general, it appears that the refined PUFA model designed here was an improvement on that in the original study. Even in the short-term, substantial positive effects on adiposity and lipid metabolism were observed, and the stimulation of energy expenditure suggested, in response to a high intake of the omega-3 FA, DHA. Whether its benefits are expressed behaviourally may require further work over longer periods of dietary exposure. This would also answer the question regarding whether or not the benefits observed are sustained. That this model showed changes equally in relation to the low-fat-fed control group and an isocaloric control group, indicates that the changes seen were attributable to the omega-3 FAs, not to differences in dietary energy content. As the changes were towards a lean, rather than obese phenotype, and moreover, below normal, it could be argued that the isoenergetic control was unnecessary. This is perhaps just as well, given the issue of suitability of coconut oil as an SFA source for obesity induction. The interesting question then becomes whether the high-DHA diet would prevent or reverse the detrimental effects of an effective high-SFA diet, perhaps the original, lard-based one, or perhaps one containing coconut oil, but allowed to be consumed for longer (Buchner *et al.,* 2008; Lee *et al.,* 2008).

Thus, it could now be concluded with greater confidence that chronic consumption of a PUFA-enriched diet would improve energy metabolism. However, this would require confirmation over more prolonged feeding periods and inclusion of additional measures, such as adipokine and insulin levels in the blood. This model could then be used to investigate changes in the hypothalamus, including cell proliferation, which underpin these responses. Initial tests in this area are presented and discussed in Chapter 7.

# CHAPTER 6

## STIMULATION AND OBSERVATION OF HYPOTHALAMIC CELL PROLIFERATION

# Stimulation and Observation of Hypothalamic Cell Proliferation

## 6.1 Introduction

### 6.1.1 Hypothalamic Neurogenesis

Until recently, neurogenesis was thought to occur only in the embryo, but it is now also known to occur in the adult mammalian brain. The regions which show it include the subventricular zone (SVZ), dentate gyrus (DG) of the hippocampus (Jessberger & Gage, 2008; Miguad *et al.,* 2010) and most recently, the hypothalamus (Kokoeva *et al*., 2007; Migaud *et al.,* 2010; Yuan & Arias-Carrion, 2011) and circumventricular organs (Bennett *et al.,* 2009). A variety of influences stimulate neurogenesis, ranging from the metabolic (e.g. diet, exercise) (Lindqvist *et al.,* 2006; Rafalski & Brunet, 2011) to the environmental (e.g. scent communication, enrichment of surroundings) (Kempermann *et al.,* 1997; Mak *et al.,* 2007).

The role of hypothalamic nuclei in regulating appetite and body weight is well-established. These nuclei include the appetite centre of the brain, the lateral hypothalamic area (LHA), and the satiety centre, the ventromedial hypothalamus (VMH). The arcuate (ARC) and paraventricular nuclei (PVN) also play critical roles in regulating appetite (Schwartz *et al.,* 2000; Marx, 2003). The third ventricular cavity (3V), which is located between these feeding related nuclei in the hypothalamus, has an ependymal lining comprised of a single layer of cells, some of which extend long processes into the hypothalamic parenchyma. These are known as tanycytes (Flament-Durand & Brion, 1985) and are morphologically similar to embryonic radial glia cells, which, in turn, have been regarded as similar to stem cells in the developing and adult central nervous system (Tamamaki *et al*., 2001; Antony *et al*., 2004; Mathew, 2008; Lee *et al.,* 2012). These discoveries have prompted research which has confirmed that there are stem cells in the ependymal lining of the adult 3V which may originate from tanycytes, and that then migrate to the hypothalamic parenchyma, to become functional neurons in the adult rat (Xu *et al*., 2005; Mathew, 2008). These findings have substantiated the hypothalamus as a neurogenic niche.

Mice with diet-induced obesity (DIO) show reduced hypothalamic cell proliferation (Kokoeva *et al.,* 2005), but whether this is due primarily to elevated

concentrations of saturated fatty acids (SFAs) in the diet is unknown. These animals also show sustained weight loss when hypothalamic neurogenesis is stimulated by central administration of ciliary neurotrophic factor (CNTF). The fate of these proliferating cells, tracked by co-administration of bromodeoxyuridine (BrdU), is such that they differentiate into neurons and integrate into appetite-related hypothalamic nuclei, expressing the feeding-related neuropeptides, neuropeptide Y (NPY) and agouti-related peptide (AgRP). Indeed, inhibition of cell proliferation by administration of the mitotic inhibitor, Ara-C, reverses these effects. Thus, new neurons in the mouse hypothalamus become functionally mature and may play a role in body weight regulation (Kokoeva *et al.,* 2005).

### 6.1.2 Observing Hypothalamic Neurogenesis *in vivo*

BrdU has been used in numerous studies of *in vivo* cell proliferation and neurogenesis (Abrous *et al.,* 2005; Taupin *et al.,* 2007). The administration of BrdU in adult rats has revealed high levels of mitotic activity in the 3V, including amongst tanycytes in the ependymal lining. It has allowed the fate of new neurons to be tracked, such that it is now known that they are integrated into the hypothalamic neural system by the formation of synapses and generation of neuropeptides (Xu *et al.,* 2005). BrdU is traditionally delivered peripherally, by injection into the peritoneum (intraperitoneally, i.p.). Because i.p.-injected BrdU has to enter the circulation and cross the blood-brain barrier (BBB) before reaching its central targets, its concentrations may be insufficient, at least at certain sites, to be detectably incorporated into the DNA of dividing cells (Kokoeva *et al.,* 2007; Cifuentes *et al*., 2011). Studies have assessed the suitability of central BrdU delivery to better capture the proliferative potency of the adult hypothalamus, a brain region distant from the principal neurogenic sites (Koekova *et al.* 2007; Cifuentes *et al.,* 2011). It was found that the number of newborn cells detected in the hypothalamus was much higher after intracerebroventricular (i.c.v.) infusions of BrdU than after i.p. injections. Extended BrdU exposure methods, such as i.c.v. infusion (Kokoeva *et al*., 2007; Cifuentes *et al.*, 2011) and incorporation into drinking water (Zhao *et al*., 2003; Bennett *et al.,* 2009), are often required when studying adult brain sites with a generally low mitogenic activity.

Both of these infusion methods were tested in studies carried out prior to those discussed here (Yon & Pickavance, unpublished observations). The drinking water method was found to be at least as effective as invasive approaches, despite potential issues surrounding transport and integrity of the tracer. As alluded to above, it is unknown whether BrdU is degraded in the gut, and whether its crossing of the gut lining or BBB are compromised, affecting the final concentration which reaches the brain tissue. These issues may be offset to an extent, however, by the increased frequency of administration which would effectively occur with this mode of delivery. In contrast, i.p. administration is usually carried out twice a day. This approach is also more compatible with other experimental manipulations, as it prevents the need for animal handling or surgical intervention, thereby minimising stress to the animal.

### 6.1.3 Stimulation of Neurogenesis by Environmental Enrichment

The functional significance of neurogenesis is still debated, but its occurrence in the hippocampus is associated with improved memory and spatial learning in laboratory rodents, in response to complex environmental enrichment. This usually includes multiple simultaneous interventions: toys, food treats and shared housing (Kempermann *et al.,* 1997, 2002; Mak *et al.,* 2007). These confounding factors mean the adoption of such an approach presents difficulties for scientists restricted to single-housing paradigms requiring a tidy cage environment (e.g. diet intervention studies). These factors also make it complicated to determine the independent contribution of each enrichment tool to the resulting neurogenesis.

Under typical laboratory conditions, neurogenesis is minimal even in the hippocampus, a known region of rapidly dividing cells, and a stimulus appears to be required (Kempermann *et al.,* 1997). This has been substantiated in-house, in studies carried out previously in singly housed male Wistar rats; in which baseline levels of proliferation were observed to be minimal (Yon & Pickavance, unpublished observations). In contrast, the endogenous cell cycle protein Ki-67 commonly used as a marker of cell proliferation is abundantly expressed in nuclei of the hippocampi of squirrels living wild, but it could be argued that these animals were actively using this structure to access memory of food caches (Amrein *et al.,* 2004). The likelihood of this is further supported by findings that the induction of long-term potentiation

(signal transmission between two neurons that results from stimulating them synchronously) in the rat DG enhances proliferation of neural progenitor cells (Chun *et al.,* 2006). Long-term potentiation is one of the major mechanisms that underpin learning and memory (Bliss & Collingridge, 1993; Cooke & Bliss, 2006). In-house preliminary investigations showed no significant change in Ki-67 expression in the brains of rats with DIO, compared to chow-fed controls, suggesting that a novel diet alone might not be a sufficient neurogenic stimulus (Yon & Pickavance, unpublished observations). In feeding studies, the laboratory rodent's cage environment is typically impoverished and neurogenesis must be induced by stimulating tasks, or trophic or pharmaceutical intervention (Kokoeva *et al.,* 2005).

As feeding studies require individual housing and an uncluttered environment, a non-invasive method, such as a simple enrichment, by which to stimulate neurogenesis is preferable. Furthermore, if levels of neurogenesis could be raised by the presence of such a simple enrichment in the cage environment, would this intervention necessarily synergise with the dietary stimulus to raise neurogenic levels above this new 'baseline'?

### 6.1.4 Pilot Study Findings

In order to address some of the questions raised, a pilot study ($n$=1/group) was conducted to investigate whether a non-invasive approach might achieve the same results as trophic intervention. The addition of a simple play tube to the home cage of each laboratory rodent, to act as an environmental enrichment factor, was of particular interest. This was because dietary intervention studies often require sparse cage environments and single-housing paradigms that make it difficult to provide sufficient enrichment without introducing variability that can confound results. This study showed, for the first time, that a PVC play tube placed in the cages of singly housed rats succeeds in dramatically stimulating neurogenesis, not only in the hippocampus, but also in the hypothalamus. These findings required further confirmation and were expanded upon in the study detailed here.

Evidence of a neurobiological basis for welfare improvements in laboratory rodents, exposed to simple environmental enrichment, is lacking and would encourage more widespread take-up of enrichment approaches by scientists. Complex enrichment, which stimulates neurogenesis in group-housed mice

(Kempermann *et al.,* 1997), is not possible where single-housing and uncluttered cage environments are required (e.g. feeding studies). The findings of such a study would also take a step toward mimicking more natural conditions in these otherwise environmentally compromised studies, thereby leading to more realistic and easily interpretable results in animal modelling studies.

### 6.1.5 Relevant Circulating Factors

Exposure to non-threatening environments is now also thought to lead to increased neurogenesis (Olson *et al*., 2006), whereas animals exposed to stressful situations show a rapid decrease in cell proliferation (Warner-Schmidt & Duman, 2006). Corticosterone (CORT) is a steroid hormone produced in the adrenal glands and involved in the control of stress. Recent studies have suggested that environmental enrichment can lessen negative physiological responses to stressful situations (Belz *et al.,* 2003; Fox *et al*., 2006) and therefore, reduce circulating concentrations of CORT (Belz *et al*., 2003). Social isolation is also considered a stressor for rodents, as they are commonly group-housed, and therefore, this may also affect levels of cell proliferation in the brain (Wu & Wang, 2010). In order to investigate these effects, plasma concentrations of circulating CORT were measured.

Brain-derived neurotrophic factor (BDNF) is a neurotrophin which promotes neuronal differentiation, survival during early development, adult neurogenesis, and neural plasticity (Chao *et al.,* 2006; Noble *et al.,* 2011; Ooi *et al.,* 2012). BDNF is expressed and present in high circulating concentrations in the hippocampus and cerebral cortex of the brain. BDNF and its receptor, TrkB, are also extensively expressed in neurons of the hypothalamus (Wu *et al.,* 2004a; Wu *et al.,* 2004b; Webster *et al.,* 2006; Godar *et al.,* 2011; Noble *et al.,* 2011). The involvement of BDNF in neural plasticity and neurogenesis within the hippocampus is known to influence learning and memory, but less is known about how it influences hypothalamic neurogenesis (Noble *et al.,* 2011). In order to investigate whether circulating concentrations of BDNF would correlate with cell proliferation, to act as an easy-to-measure surrogate marker for future studies, concentrations of circulating BDNF were measured here.

### 6.1.6 Research Question

***Is a simple physical enrichment tool sufficient to stimulate central cell proliferation in singly housed rats?***

### 6.1.7 Pilot Study

#### 6.1.7.1 Aims

To address this question by assessing the quantity of proliferating cells observed in central neurogenic niches of rats housed with a single play tube each, compared to those without play tubes.

#### 6.1.7.2 Expected Outcomes

In rats exposed to play tubes, enhanced cell proliferation would be observed in the hypothalamus and hippocampus.

### 6.1.8 Full Study

#### 6.1.8.1 Aims

1. To statistically confirm the above findings, and
2. To test whether any observed reduction in cell proliferation over time was due to habituation to the enrichment tool.

#### 6.1.8.2 Expected Outcomes

In rats exposed to play tubes,

1. enhanced cell proliferation in the hypothalamus and hippocampus was a reproducible finding, and
2. proliferation levels which had fallen as a result of habituation would be restored by introduction of a new tube (stimulus renewal).

A technical issue with this project aim was raised early on: concurrent attempts to optimise the method uncovered difficulties in dual-labelling of BrdU and neuronal markers; thus, within the time constraints of the project, only cell proliferation was examined (not neurogenesis *per se*).

## 6.2 Materials & Methods

### 6.2.1 Animals and Treatment

Age-matched adult male Wistar rats (~250 g) were used in both pilot and full studies. In the pilot study, a PVC play tube was introduced to the cage of an experimental rat, but not to that of its control ('+tube' *vs.* '-tube'; *n*=1/group), for 7 days, while BrdU (Sigma, Dorset, UK) was administered in the drinking water (1 mg/ml). Rats were perfuse-fixed under deep anaesthesia and brains dissected free for histological processing.

In the full study, rats were stratified by body weight and randomly assigned to six groups (*n*=6/group) (Fig. 6.1). Tube exposure periods of 7, 21 and 28 days were selected to examine the time course of effects of simple physical enrichment on cell proliferation and neurogenesis in the proliferative regions observed in the pilot study (3V/hypothalamus and DG). Exposure periods would also enable observation of both proliferating cells (7 days), young (21 days) and mature neurons (28 days). Acute (7-day) exposure was included to determine if pilot findings (increased proliferation in response to enrichment; Fig. 6.2) could be replicated and if so, increased sample size would allow for statistical analysis. This could then be compared to the effects of chronic (21-day) exposure, where it was predicted that proliferation might be reduced, as a result of habituation to the tube. A link between reduced neurogenesis and habituation to neurogenic stimuli has been shown previously (Veyrac *et al.,* 2009), and was therefore suspected as a possible reason for conflicting results in animals examined concurrently with pilot animals, in which proliferation was minimal after exposure to tubes for 8 weeks (diet study described in Chapter 7). Therefore, the introduction of a fresh tube (renewal of stimulus) at 21 days, for an additional 7 days, was included as an experimental group (Group 6; see Fig 6.1) to investigate whether the new stimulus restored neurogenic levels. Olfactory stimuli from rodent con-specifics are well-known to influence neurogenesis (Mak *et al.,* 2007; Veyrac *et al.,* 2009). Therefore, tubes were first cleaned to eliminate any previous animal scent, which could confound results by acting as an additional form of enrichment and therefore, stimulus to neurogenesis.

Body weight was measured weekly, and food and water intake daily, to monitor any changes in health as a result of BrdU consumption. Rats were perfuse-

fixed and brains removed, as for the pilot study. Spleens were also removed to provide positive control tissue for BrdU uptake. Details of general husbandry and maintenance and termination and tissue harvesting protocols are further detailed in Chapter 2, Sections 2.1.6 and 2.5.

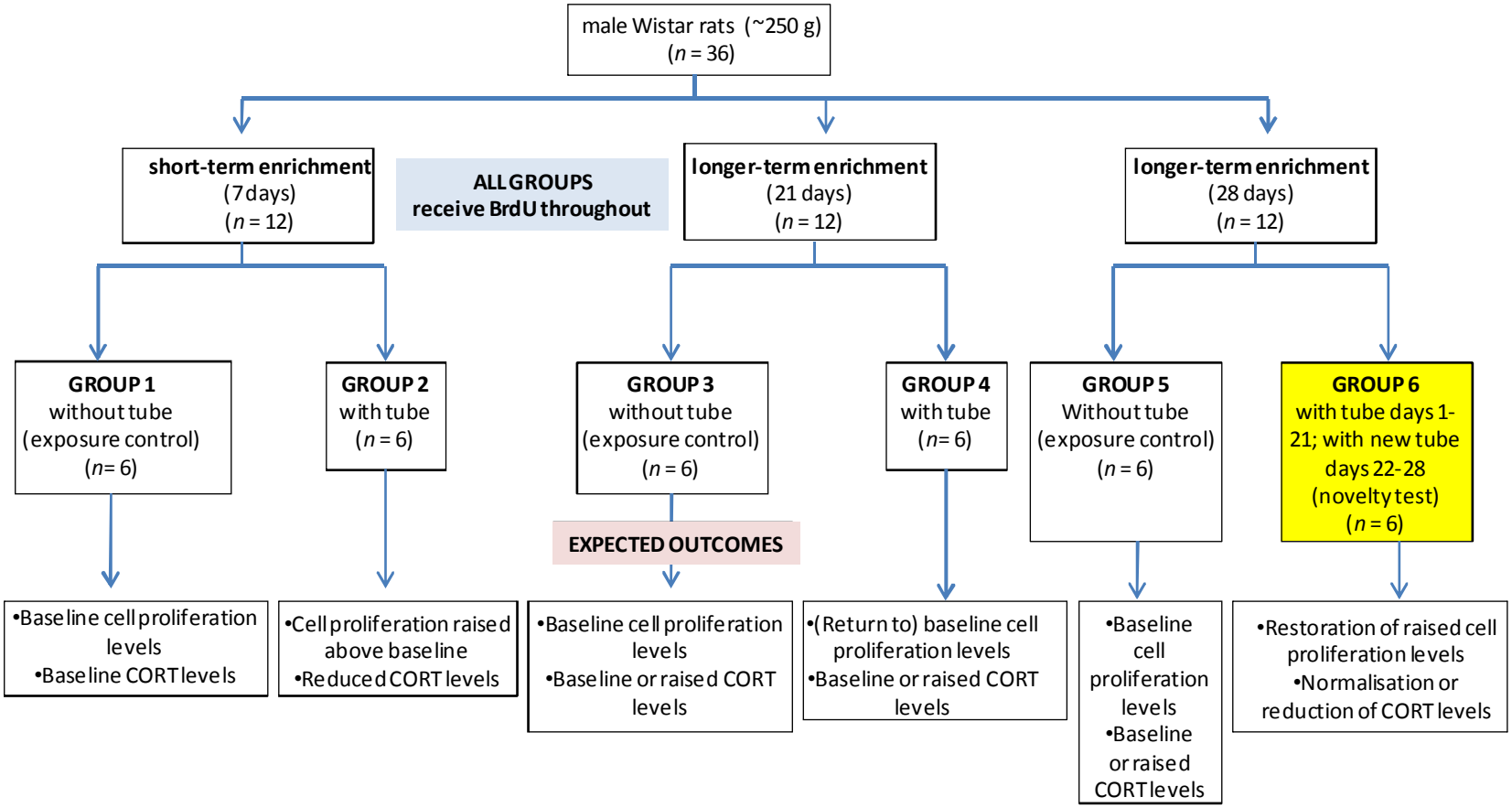


**Figure 6.1. Study design and expected outcomes for effects of simple physical enrichment on cell proliferation**. Blood sampling was kept to a minimum (terminal sample only), to avoid unnecessary stress effects on neurogenesis. Plasma concentrations of corticosterone (CORT) were measured as an indirect index of stress. It was predicted that the stress of prolonged social isolation of single-housing in the 21-day groups might elevate CORT concentrations, compared to 7-day isolation. At study end, animals were perfuse-fixed and it was planned that brains would be examined between groups for changes in immunohistochemical evidence of cell proliferation (BrdU+) and new neurons (DCX+). Terminal plasma concentrations of BDNF were also determined, as a potential surrogate marker of neurogenesis.

### 6.2.2 Blood Chemistry

Repeated blood sampling was avoided, as stress is known to inhibit neurogenesis (Fox *et al.,* 2006; Warner-Schmidt & Duman, 2006). After an overnight fast, and under brief gaseous anaesthesia, blood samples were collected from the tail vein and plasma separated by centrifugation. As an indirect measure of stress, possibly arising from prolonged social isolation (Stranahan *et al.,* 2006), plasma concentrations of corticosterone (CORT), also known to be associated with alterations in neurogenesis (Olson *et al*., 2006), were determined. BDNF concentrations were also measured to determine whether they might correlate with neurogenic levels (Chao *et al.,* 2006; Noble *et al.,* 2011; Ooi *et al.,* 2012). Both were measured by ELISA, according to manufacturers' protocols, as detailed in Chapter 2, location 2.6.

### 6.2.3 Histology

For both studies, after a three-hour post-fixation and overnight immersion in cryoprotectant at 4°C, hypothalamic blocks isolated from brain were sectioned at a thickness of 50 μm on a freezing microtome and processed free-floating. Spleen tissue, a positive peripheral control for cell proliferation, was processed identically throughout and at the same time. Brain sections were collected throughout the 3V/hypothalamic region and the hippocampus, within which the DG acted as a positive internal control for cell proliferation. Prior to immunolabelling, sections underwent an acid pre-treatment to denature the DNA. They were incubated in 1M HCl for 30 minutes at 37°C to allow antibody access to BrdU incorporated into DNA. The acid was then neutralised by rinsing the sections three times in 10 mM PBS with constant agitation (adapted from Wojtowicz & Kee, 2006).

Investigations of cell proliferation were carried out using an antibody raised against BrdU. Though proliferation was extensive in the pilot study, it was scant in the full study (Figs. 6.2 & 6.3). To confirm whether this was due to failure of BrdU uptake, antibodies raised against endogenous markers of cell proliferation (Ki-67 and PCNA) were applied to adjacent sections. Primary antibody incubations were carried out with mouse anti-BrdU (1:50; Roche), rabbit anti-Ki-67 (1:100; Vector Labs) or mouse anti-PCNA (1:100; Santa Cruz) at 4°C overnight. This was followed by one-hour incubations with red fluorophore-conjugated secondary antibodies raised in appropriate host species [DyLight 594 (1:500); Jackson ImmunoResearch

Laboratories] at room temperature in the dark. Sections were washed in 10 mM PBS between each step and finally mounted onto chrome-alum-coated glass slides. After air drying overnight, anti-fade reagent (Vector Laboratories, Burlingame, USA) and coverslips were applied. Full details of antibodies and staining procedures are given in Chapter 2, Section 2.10.4.

### 6.2.4 Image Analysis

Immunostaining was examined under an epi-fluorescent microscope (Zeiss Axio Imager. M1), and images captured by digital microphotography (Hamamatsu ORCA I-ER digital camera, Hamamatsu Photonics, Welwyn Garden City, Herts) and image analysis program (AxioVision, Zeiss Imaging Systems). Photographic panels were created using Microsoft PowerPoint 2007.

### 6.2.5 Statistical Analysis

Data are expressed as mean ± SEM. Differences between groups over a time course were determined using 2-way ANOVA with repeated measures and post-hoc Bonferroni correction and considered significant at $p<0.05$. In all instances comparisons demonstrated roughly equal variation and therefore the sphericity criteria were met and consequently variation data is not presented here. Statistical analysis was carried out using software SPSS *v*19. Justification for use of all statistical analyses can be found in Chapter 2 section 2.11.

## 6.3 Results

### 6.3.1 Pilot Study

Seven days of simple environmental enrichment with a single PVC play tube (+ tube) dramatically increased cell proliferation in the lateral wall lining of the third ventricle, as well as in the adjoining medial hypothalamic parenchyma (Figs. 6.2A&B; 9±2 *vs.* 82±6 cells per field of view). Proliferation was also markedly increased in the dentate gyrus of the hippocampus (Figs. 6.2C&D; 3±3 *vs.* 68±9 cells per field of view). As this is an inherently proliferative region, it was investigated as a positive internal control. Extensive BrdU immunoreactivity here indicated, therefore, that BrdU uptake into the brain had been successful and staining conditions optimal, validating expression in the hypothalamic region.

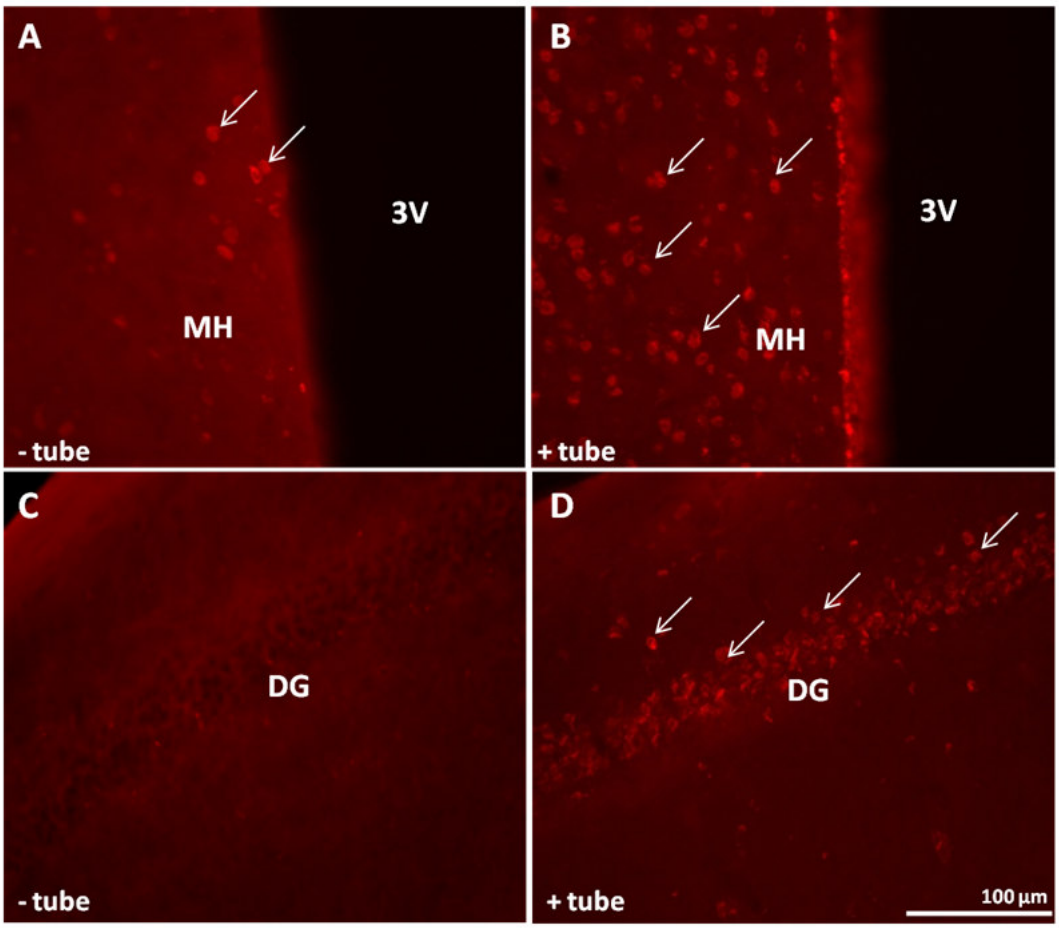


**Figure. 6.2. Pilot study.** Representative photomicrographs of cell proliferation in response to 7-day cage enrichment with a play tube ('+tube'). Control rats did not receive a tube ('-tube'; *n*=1/group). Proliferating cells show BrdU-positive immunoreactivity in cell nuclei (red fluorescent profiles; examples indicated by arrows). **Extensive proliferation was stimulated by tube presence** in the lateral wall 3V lining, medial hypothalamus (A, B) and dentate gyrus of the hippocampus (positive control; C, D). **Abbreviations:** 3V = third ventricle, DG = dentate gyrus, MH = medial hypothalamus.

### 6.3.2 Full Study

In contrast to findings in the pilot study, the addition of a play tube to rat cages for 7, 21 or 28 days did not stimulate cell proliferation in hypothalamic or hippocampal regions (Figs. 6.3A-D). Positive BrdU immunoreactivity was observed in the roof of the third ventricle, but this was very sparse, occurring to a similar minimal extent in all exposure groups, irrespective of tube presence (Fig. 6.4A, D). It was also observed in nuclei of epithelial cells comprising the *pia mater*, lining the outside surface of brain sections. This confirmed that BrdU had been administered in sufficient concentration that a high-turnover tissue in very close proximity to the brain had taken up the tracer, and that anti-BrdU immunolabelling had been successful. However, it did not prove that BrdU had crossed the BBB, and therefore, confirmation was sought that the lack of specific staining reflected a real absence of proliferation. Thus, immunoreactivity against the endogenous proliferation markers, Ki-67 and PCNA, was examined, but also found to be equally scant in all groups (Figs. 6.4B, C, E & F). Spleen tissue, the cells of which are also quickly self-replicating, was examined as an additional positive control. Specific staining with all antibodies in this tissue indicated the success of peripheral BrdU uptake and the adequacy of the immunohistochemical technique as a whole.

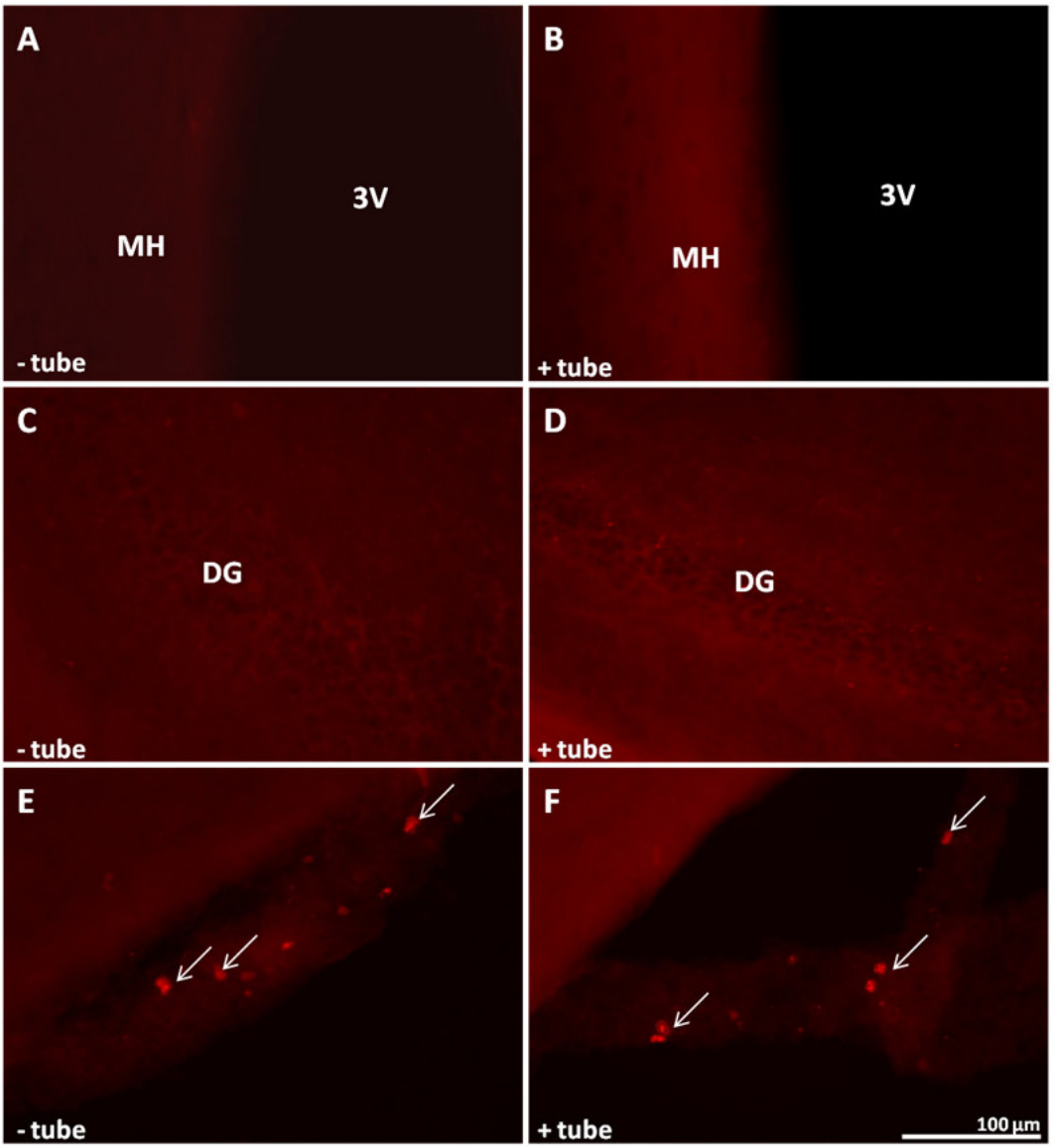


**Figure. 6.3**. **Full study.** Representative photomicrographs of time course of cell proliferation in response to cage enrichment with a play tube ('+tube'), in regions matching those observed in the pilot study. Control rats did not receive a tube ('-tube'*; n*=6/group). As all periods of tube exposure (7, 21 and 28 days) produced similar results, only photos of 7-day findings are presented. Proliferating cells show BrdU-positive immunoreactivity in cell nuclei (red fluorescent profiles; examples indicated by arrows). **In contrast to pilot study findings, tube presence failed to stimulate proliferation** in the lateral wall 3V lining and medial hypothalamus (A, B) or dentate gyrus of the hippocampus (positive control; C, D). BrdU uptake was confirmed, however, in the *pia mater* [(E, F); positive control]. **Abbreviations:** 3V = third ventricle, DG = dentate gyrus, MH = medial hypothalamus.

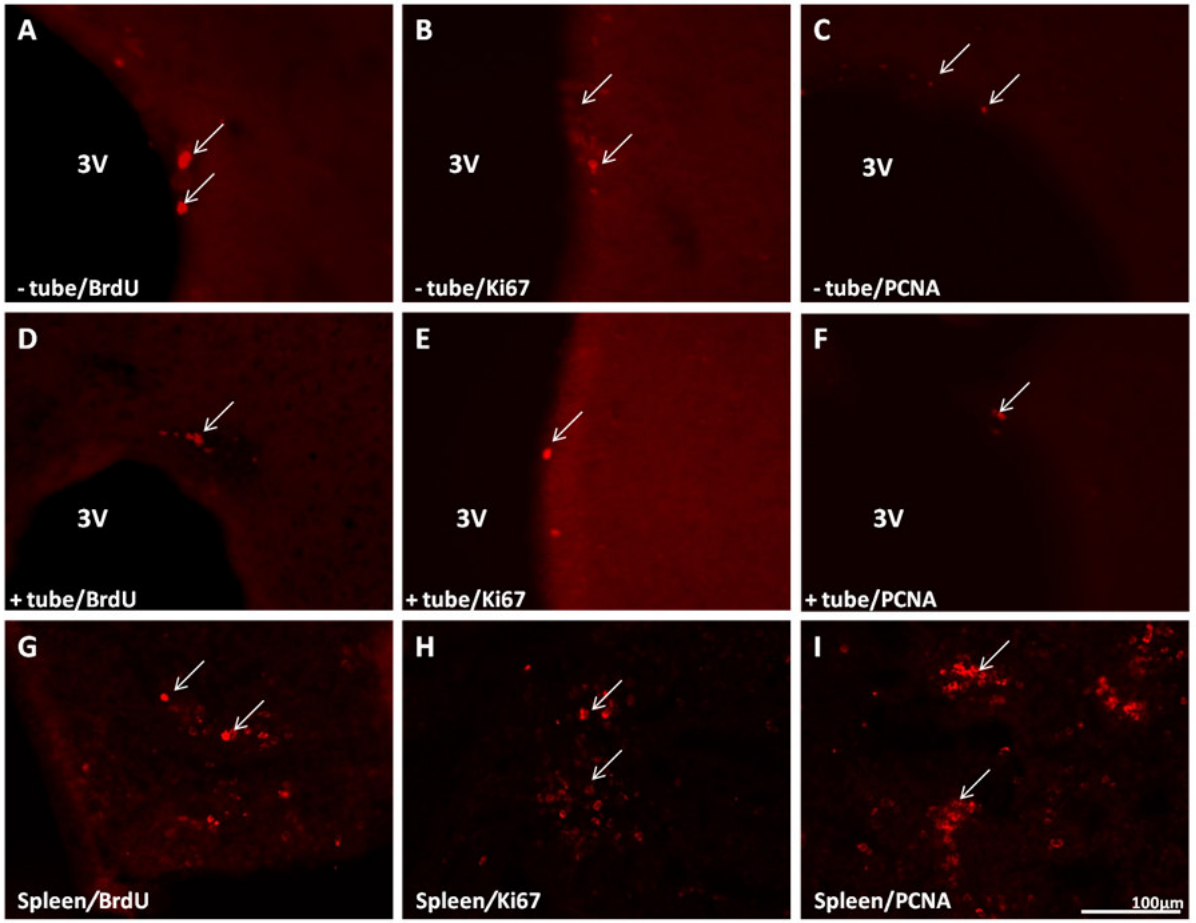


**Figure. 6.4**. **Full study.** Representative photomicrographs of cell proliferation survey in response to cage enrichment with a play tube ('+tube'). Control rats did not receive a tube ('-tube'*; n*=6/group). As all periods of tube exposure (7, 21 and 28 days) produced similar results, only photos of 7-day findings are presented. Proliferating cells show BrdU- (A, D), Ki-67- (B, E) and PCNA-positive immunoreactivity (C, F) in cell nuclei (examples indicated by arrows). **Although sparse proliferation observed as BrdU-positive cells was confirmed by endogenous markers Ki-67 and PCNA, none indicated that tube presence had a stimulatory effect.** BrdU uptake and proliferation in general were confirmed, however, in spleen (G, H, I; peripheral positive control tissue).

Analysis by two-way ANOVA with repeated measures demonstrated no differences in circulating CORT concentrations between groups ($F(1, 10) = 0.4400$, $p=0.5221$), over time ($F(2, 20) = 2.556$, $p=0.1027$) or any interaction between enrichment and time ($F(2, 20) = 1.801$, $p=0.1909$). Therefore, the presence of the play tube had no effect on circulating CORT concentrations at any of the exposure times examined ($p>0.05$; Fig. 6.5).

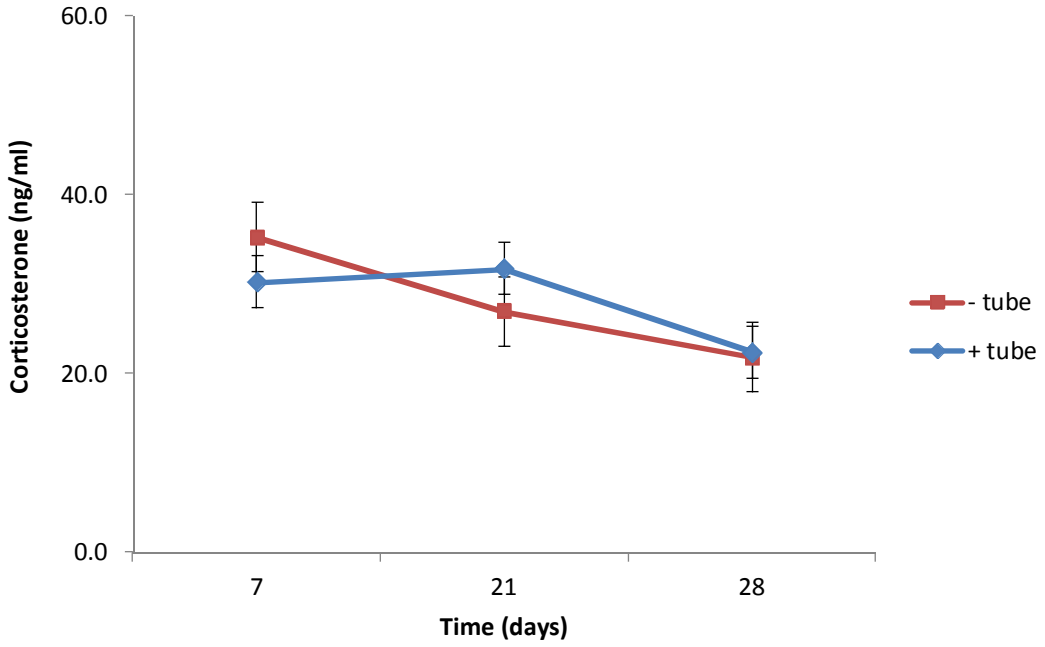


**Figure 6.5.** Time course of plasma corticosterone concentrations in rats with or without cage enrichment in the form of a play tube (+tube). **There was no effect of tube exposure at 7, 21 or 28 days.** Values are expressed as mean ± SEM; $n=6$/group; all $p>0.05$ compared to controls (-tube) (2-Way ANOVA with repeated measures).

Analysis by two-way ANOVA with repeated measures demonstrated no differences in circulating BDNF concentrations between groups ($F(1, 10) = 0.05642$, $p=0.8170$) or any interaction between enrichment and time ($F(2, 20) = 0.4287$, $p=0.6572$), but it did detect a difference over time ($F(2, 20) = 4.649$, $p=0.0220$). Therefore, the presence of the play tube had no effect on circulating BDNF concentrations but BDNF concentrations in both groups increased over time irrespective of enrichment ($p>0.05$; Fig. 6.6).

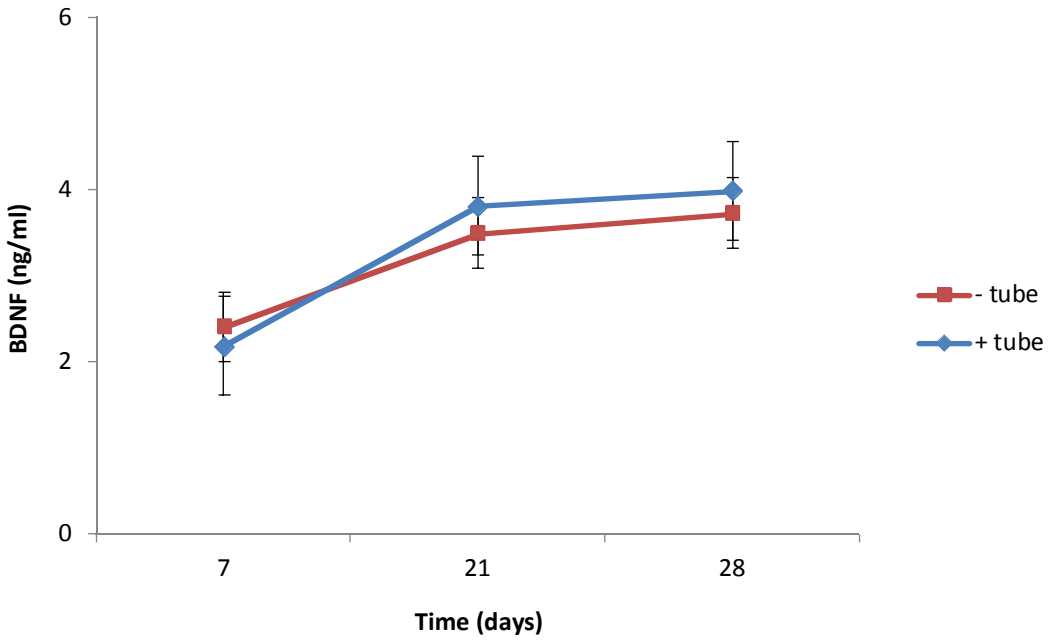


**Figure 6.6.** Time course plasma BDNF concentrations in rats with or without cage enrichment in the form of a play tube (+tube). **There was no effect of tube exposure at 7, 21 or 28 days.** Values are expressed as mean ± SEM; $n=6$/group; all $p>0.05$ compared to controls (-tube) (2-Way ANOVA with repeated measures).

## 6.4 Discussion

### 6.4.1 Pilot Study

The pilot study described here demonstrated that simple environmental enrichment in the form of a play tube can stimulate cell proliferation within regions of the medial hypothalamus and lining of the third ventricle, regions of slow cell turnover, as well as in the dentate gyrus of the hippocampus, a prolific site, in singly housed rats. Assuming that adult neurogenesis is beneficial, this novel result provides the first evidence that improvements to laboratory rodent welfare are reflected at the cellular level. Nonetheless, this result was somewhat surprising, as the literature suggests that to stimulate cell proliferation in the rodent hypothalamus or hippocampus, a trophic or pharmaceutical intervention may be required (Yoshimizu & Chaki, 2004; Kokoeva *et al.,* 2005). In addition, the findings were treated with caution, as only a single experimental and control animal had been tested. In order to corroborate the findings of the pilot study, a full study with appropriate statistical power was conducted.

### 6.4.2 Full Study

In contrast to findings in the pilot study, the addition of a play tube to rat cages for 7, 21 and 28 days had no effect on cell proliferation in the medial hypothalamus, lining of the third ventricle or dentate gyrus. Positive immunoreactivity in control tissues confirmed that BrdU had been successfully taken up, and that immunohistochemical techniques had worked. Therefore, the general absence of BrdU-positive cells in regions of interest demonstrated that the play tube was an insufficient stimulus to cell proliferation here and therefore, that the findings of the pilot study were not reproducible. As such, the additional investigations planned to test for any observed reduction in cell proliferation over time could not be answered. In hindsight, it would have been useful to have collected spleen and *pia mater* from animals in the pilot study to determine the levels of proliferation that could have been expected under successfully stimulated conditions; i.e. it could be that although there was more proliferation here in positive control tissues than in regions of interest, the extent of staining did not reflect 'normal' levels resulting from optimal BrdU uptake. However, given the striking results in the pilot study, this precaution had not been thought necessary. The inability to reproduce the findings of the pilot study may be

due to the rats being of an outbred strain, with inherent genetic variation, as discussed in Chapter 3, Section 3.5 (Koolhass, 2010).

Seemingly paradoxically, some studies have shown that there is reduced cell proliferation in the brains of mice exposed to more complex enrichment. In the work of Veyrac *et al.* (2009), this involved the use of odours in a tea ball suspended from the cage lid and replaced every 24 hours. This reduction was likely due to habituation. This evidence supports the likelihood that the play tube alone was insufficient in enriching the cage environment in such a way that influenced central proliferation. A variety of additional items, including tubes, wheels and materials (paper and cardboard) would need to be added to do so (Kempermann *et al.,* 1997; van Praag *et al*., 2000; Madroñal *et al.,* 2010), even before considering habituation. Environmental enrichment is commonly defined as a mixture of complex inanimate and social stimulation (Rosenzweig *et al.,* 1978), including housing in groups, large and complex cage systems, and a variety of objects. All of this combined is thought to increase hippocampal neurogenesis (Kempermann *et al.,* 1997; Nilsson *et al.,* 1999; van Praag *et al*., 2000; Kempermann *et al.,* 2002; Olson *et al.,* 2006) relative to standard housing conditions. The addition of a running wheel to the cage has been associated with increased numbers of neurons within the DG of adult rats (van Praag *et al.,* 1999a; van Praag *et al,* 1999b; Farmer *et al.,* 2004). Studies have shown that social isolation will delay the positive effects on adult neurogenesis caused by the addition of a running wheel. In one particular study, enhancement of neurogenesis eventually occurred in individually housed runners, but only after a considerably longer time period than for group-housed animals (48 days instead of 12) (Stranahan *et al*., 2006). A 'control' group of rats given extensive environmental enrichment could have been used in the current study to establish a sense of scale when looking at the effect of environmental enrichment on cell proliferation.

### 6.4.3 Circulating CORT Concentrations

Circulating concentrations of CORT were examined as a simple marker of anxiety/stress (De Souza & Loon, 1982). Had reductions in proliferation been observed at any point or in any group, this would have suggested an association between the two phenomena, as indeed, stress is known to inhibit neurogenesis (Fox *et al.,* 2006; Warner-Schmidt & Duman, 2006). Irrespective of the presence of

enrichment all animals groups at all time points with and without a play tube showed similar concentrations of circulating CORT.

Recent studies have suggested that environmental enrichment can lessen negative physiological responses to stressful situations (Belz *et al.,* 2003; Fox *et al*., 2006). Studies have shown that rats housed with environmental enrichment in the form of rubber toys and nest materials have lower plasma CORT concentrations than those housed without (Belz *et al*., 2003). Stress responses are mediated by the hypothalamic-pituitary-adrenal (HPA) axis, and enrichment is thought to decrease activity of the HPA and therefore, to decrease CORT concentrations (Warner-Schmidt & Duman, 2006). Exposure to non-threatening environments is now also thought to lead to increased cell proliferation in the hippocampus (Olson *et al*., 2006), whereas animals exposed to stressful situations show a rapid decrease in cell proliferation (Warner-Schmidt & Duman, 2006). However, as CORT concentrations were unchanged here in rats with play tubes, it may be that this intervention is not sufficient to reduce stress, or that all the animals, including controls, were already experiencing minimal stress possibly as a result of single housing. Although this result is supported by some findings in the literature, it directly opposes that of others, and the current consensus is that results are inconclusive (Smith & Corrow, 2005). This is mainly due to the extreme variability in enrichment methods that are carried out in different research groups (Sztainberg & Chen, 2010). Several groups have provided animals with wheels to encourage exercise, which in turn, has reduced baseline CORT concentrations (Olsson & Dahlborn, 2002), as well as hippocampal neurogenesis (Kobilo, *et al.,* 2011). This further supports the idea that the play tube alone was insufficient enrichment. Rats kept in enriched environments can have higher resting plasma CORT concentrations at the first instance of handling, followed by their rapid extinction upon exposure to repeated handling (Moneck *et al.,* 2004). Although there was minimal handling involved in this study (e.g. for maintenance only), it is possible that this, and the daily interaction when weighing food and water for health assessment, was enough to induce this extinction response, as stress responses have been shown to recover rapidly after brief handling periods, causing a reduction in sensitivity to parameters such as CORT concentrations (Konkle *et al.,* 2010). One study suggested that if rats were kept in a stress-free environment, the presence of environmental enrichment would have no link with

physiological status, such as those reflected in CORT concentrations (Morley-Fletcher *et al.,* 2003). The lack of effect observed here suggests that environmental enrichment may only be important in recovery from acute stressful stimuli (Benaroya-Milshtein, *et al*., 2004; Moncek, *et al*., 2004). An additional point for consideration is the use of the Wistar rat strain in this particular study. A review of existing literature reveals that there have been few behavioural investigations into the effects of environmental enrichment on stress responses. The few that have been carried out have favoured the use of Sprague-Dawley and Long Evans rats over the Wistar, and have produced inconsistent results at best (Konkle *et al.,* 2010). Wistars may have been avoided in these studies because they are known to be an inherently anxious strain (Simpson & Kelly, 2011); a characteristic which could explain generally inhibited cell proliferation, although the normal CORT levels observed would dispute this. Some studies have shown no differences in CORT concentrations for Long-Evans and Lister Hooded rat strains housed in standard and environmentally enriched conditions after exposure to stress (Francis *et al.,* 2002; Schrijver *et al.,* 2002; Morley-Fletcher *et al.,* 2003), whereas others have shown inconsistent results under conditions of enrichment; i.e. reductions in Sprague–Dawley rats (Belz *et al.,* 2003), and increases in Wistar rats (Moncek *et al.,* 2004). These differences are likely due to strain-specific responsivity to stress. This specificity has been discovered when examining effects of enrichment on weight gain (where weight loss indicates stress): group-housed and isolated control Wistar rats showed no differences in weight gain (von Frijtag *et al.,* 2000), whereas physical and social enrichment reduced weight gain in Sprague-Dawley rats (Pena *et al.,* 2006). This suggests that Wistar rats show less sensitivity to stress, but contradicts the findings above (Simpson & Kelly, 2011). Perhaps stress responses are dependent on the interaction of strain with the specific stress stimulus. Overall, therefore, in order to assess the effects of enrichment thoroughly, future studies could be designed to examine other indices of stress apart from circulating CORT concentrations, and in several rat strains; e.g. behavioural (anxiety) tests, such as the open field or elevated plus maze test (Kulesskaya *et al.,* 2011).

### 6.4.4 Circulating BDNF Concentrations

Environmental enrichment increases the expression of several different neurotrophic factors that have been implicated in cell proliferation and neuronal differentiation

and survival, including BDNF and the corresponding receptor, TrkB (Pham *et al.,* 1999; Young *et al.,* 1999; Ickes *et al.,* 2000; Gobbo and O'Mara, 2004). Here however, there was an early gradual rise in circulating concentrations of BDNF independent of tube presence. This leads to the speculation that, as BDNF is involved in the process of learning and memory formation (Dragunow, 1996; Yamada *et al*., 2002), rodents showed an increase in these initial weeks due to exploration of their new cage environment (after transport and acclimatization) or to any other factor that both groups may have experienced that had not been controlled for, such as reduced cage size and social contact (after group housing during acclimatization), or any unidentified changes to experimental conditions.

Some research has shown that the addition of environmental enrichment, such as a running wheel, to the cage increases expression of BDNF in the brain (Neeper & Gomez-Pinilla, 1996), but that plasma concentrations are often unaffected (Vedovelli *et al*., 2011), as seen here. Studies which investigated the effects of environmental enrichment on BDNF expression in the hippocampus and serum of rats exposed to 10 weeks of standard or enriched conditions (increased opportunity for physical exercise) revealed that animals maintained in the enriched condition showed no alterations in central or peripheral BDNF concentrations (Vedovelli *et al.,* 2011). Like environmental enrichment, exercise can also increase concentrations of BDNF (Adlard *et al.,* 2004; Farmer *et al.,* 2004; Vaynman *et al.,* 2004a), as shown by the introduction of a running wheel (Neeper & Gomez-Pinilla, 1996) or enforced swim test (Badowska-Szalewska *et al.,* 2010). The level of BDNF expression is directly related to the amount of exercise carried out (Adlard *et al.,* 2004). This suggests that environmental enrichment which increases exercise (e.g. a running wheel) will promote concentrations of BDNF above those associated with other forms of enrichment.

### 6.4.5 Corticosterone & Brain-Derived Neurotrophic Factor

Physiological and pharmacological increases in plasma CORT concentrations have been shown to reduce BDNF expression and thereby, increase hippocampal neuronal loss following brain injury. Stress, or the direct administration of CORT, has been shown to decrease BDNF gene expression and circulating concentrations in the rat hippocampus (Smith *et al.,* 1995). On the other hand, the removal of circulating

CORT by adrenalectomy increases the levels of BDNF mRNA in rat hippocampus (Chao *et al.,* 1998), which is then reversed upon direct administration of CORT (Schaaf *et al.,* 1998). Therefore, these two measures are inversely related, but the mechanism behind this is, as yet, unexplained. This relationship was suggested here: as CORT concentrations decreased between days 7 and 21, BDNF concentrations increased. The chronic forced swim test (FST) combines factors such as the novelty effect of water and the physiological stressors of exercise and swimming. For rats, this represents a stressful situation that they may not frequently encounter in their natural environment. Studies have shown a decreased density of BDNF- and TrkB-immunoreactive neurons in the hippocampus of juvenile rats, which is linked with increased CORT concentrations (Badowska-Szalewska *et al.,* 2010). This study also investigated the effects of the FST on both juvenile and aged rats; however, there were no changes in the density of BDNF- or TrkB-expressing neurons in aged rats, suggesting that juvenile rats are more sensitive to stress than aged rats. The rats used in the study presented here were young adults (8 weeks old), having reached sexual maturity (Koolhaas, 2010), and therefore, may have grown beyond any such window of sensitivity.

## 6.5 Conclusion

This study aimed to address whether simple physical enrichment would stimulate cell proliferation in the hypothalamus of singly housed rats. The pilot study suggested that the presence of a play tube may be sufficient to stimulate cell proliferation in the hypothalamus and hippocampus; however, these results were non-reproducible in the full study. As a result, it was impossible to determine any effects of habituation.

There are several possible explanations for the lack of effect on cell proliferation and related circulating factors, but insufficient enrichment seems to be the most likely candidate. However, as the influences on neurogenesis are many, re-testing the original hypothesis would require the careful design of a multifactorial study to control for each of these factors individually.

# CHAPTER 7

## EFFECTS OF HIGH-PUFA FEEDING ON HYPOTHALAMIC CELL PROLIFERATION AND MYOGENESIS

# Effects of High-PUFA Feeding on Hypothalamic Cell Proliferation and Myogenesis

## 7.1 Introduction

### 7.1.1 Background

The current obesity epidemic can be largely attributed to excessive consumption of foods high in sugar (refined carbohydrates) and saturated fatty acids (SFAs), resulting in a range of cardiovascular and metabolic disorders (Kanoski & Davidson, 2011), as well as decreased neurogenesis in the brain (McNay *et al.,* 2012). These can be attenuated, if not reversed, upon consumption of polyunsaturated fatty acids (PUFAs; Poudyal *et al.,* 2011). Obesity is now increasingly being linked to depressed mood, impaired cognition and neurodegenerative diseases, such as Alzheimer's (Barnes & Yaffe, 2011). Conversely, dietary interventions with omega-3 FAs have been shown to counteract or prevent these conditions (Bourre, 2005; Hashimoto & Hossain, 2011). The use of animal models has shed some light on possible underlying mechanisms to explain these associations (Gomez-Pinilla, 2011). Adult neurogenesis has been proposed as an "interface" between energy metabolism and cognition (Vaynman & Gomez-Pinilla, 2006), but it is only recently that researchers have begun to identify the factors which may mediate this relationship; for example, changes in circulating concentrations of appetite-regulators, such as the adipokine leptin (Park *et al.,* 2011) and the neurotrophin, brain-derived neurotrophic factor (BDNF) (Bousquet *et al.,* 2009). In addition, to date, these relationships have been only examined in response to high-SFA consumption. Currently, there is no direct evidence that known neuroprotective effects of PUFAs are mediated through changes in energy metabolism.

### 7.1.2 Effects of High-Fat Diet on Neurogenesis

#### 7.1.2.1 Saturated Fatty Acids

Studies have shown that rats fed a diet high in SFAs for only nine days displayed reduced memory ability in the radial arm maze paradigm (Murray *et al*., 2009), and after only 72 hours, made significantly more memory errors when learning about spatial cues (Kanoski and Davidson, 2010). Both studies reported that memory

deficits occurred prior to the development of greater body weight gain (Murray *et al*., 2009; Kanoski and Davidson, 2010). This finding suggests that the short-term detrimental cognitive effects of consuming a diet high in SFAs can occur in the absence of differences in body weight. The results seen in these studies were underpinned by impaired hippocampal-dependent learning and memory *via* decreased cell proliferation in the same area (Kanoski and Davidson, 2011) and impaired blood-brain barrier (BBB) function (Davidson *et al.,* 2012). High-fat diet fed rats showed reduced expression of several proteins that are essential to BBB integrity and displayed elevated concentrations of a dye which is normally prevented from crossing the BBB in the hippocampus (Wolburg & Lippoldt, 2002). It was suggested that the consumption of a diet high in SFAs contributed to hippocampal inflammation, as a consequence of BBB permeability, *via* increased concentrations of peripheral proinflammatory cytokines, thus, making the hippocampus more accessible to these inflammatory agents (Fung *et al.,* 2012), as well as interfering with leptin transport across the BBB (Hsuchou *et al.,* 2010). The inability of leptin to cross the BBB under these conditions has been linked with reduced hippocampal memory and learning function (Farr *et al.,* 2006). This impairment has been attributed to insufficient concentrations of leptin for the facilitation of long-term potentiation, a process which is known to enhance cell proliferation in the hippocampus (Chun *et al.,* 2006).

Some studies have shown that rats fed a diet high in SFAs exhibit reduced cell proliferation and neurogenesis within the hippocampus (Lindqvist *et al.,* 2006; Hwang *et al.,* 2008; Park *et al.,* 2010; McNay *et al.,* 2012). In the majority of these studies, body weight data were not collected. However, even in the one study where rats displayed increased adipose accumulation in response to the high-fat diet, this was not significant and did not result in overall body weight gain; therefore, the reduction in neurogenesis was independent of adiposity and was related to dietary fat *per se* (Lindqvist *et al.,* 2006). Research has also shown that mice with diet-induced obesity (DIO) display reduced hypothalamic neurogenesis, but that central administration of ciliary neurotrophic factor (CNTF) induces cell proliferation in feeding centres, and subsequently weight loss (Kokoeva *et al.*, 2005). Furthermore, some of these newborn cells within the hypothalamus express neuronal markers and

demonstrate functional phenotypes relevant to energy homeostasis (Kokoeva *et al.*, 2005; McNay *et al.,* 2012).

#### 7.1.2.2 Polyunsaturated Fatty Acids

In contrast, it is known that animals fed a diet high in PUFAs, specifically, the omega-3 FA, docosahexaenoic acid (DHA), demonstrate an increase in cell proliferation and adult neurogenesis within the dentate gyrus of the hippocampus (Kawakita *et al*., 2006; Beltz *et al.,* 2007; Ma *et al.,* 2008; Dyall *et al.,* 2010). However, the mechanisms behind this are still not fully understood. In adult rats, learning and cognitive behaviour are linked to brain DHA status, which, in turn, is related to the concentrations of the dietary omega-3 FAs consumed (Moriguchi et *al.*, 2000). Furthermore, the administration of DHA is commonly thought to enhance learning in both young and aged rats (Gamoh *et al.,* 1999, 2001), and protects against the decline of learning and memory ability in rodent models of Alzheimer's disease (Hashimoto *et al.,* 2002; 2005; Lim *et al.,* 2005). It has also been shown to protect neurons from the effects of oxidative stress (Hossain *et al.,* 1998, 1999; Hashimoto *et al.,* 2002, 2005), although the mechanisms behind these beneficial effects have yet to be determined. As neurogenesis has been shown to play a critical role in learning and memory processes (Schinder and Gage, 2004; Becker, 2005), some studies have suggested that the beneficial effects of DHA on learning could, in part, be due to its ability to increase neurogenesis in the brain (Katakura *et al*., 2009).

Recent evidence has suggested that a link between omega-3 FAs and the BDNF/TrkB signalling pathway may explain the neuroprotective effects of omega-3 consumption in rodent models in neurodegenerative conditions (Logan, 2003; Wu *et al.,* 2004; Rao *et al*., 2007; Levant *et al.,* 2008). These protective mechanisms may include the modulation of signal transduction pathways and the reduction of inflammatory cytokines (Stoll *et al.,* 1999; Balanzá-Martínez *et al.,* 2011). The consumption of diets high in SFAs is known to decrease BDNF expression in the brain and circulating plasma concentrations (Stranahan *et al*., 2008); conversely, the consumption of PUFAs is thought to increase them (Wu *et al*., 2004; Bousquet *et al.* 2009). Furthermore, altered meal patterns such as dietary restriction can stimulate neuronal plasticity, including neurogenesis, through the stimulation of BDNF (Mattson *et al.,* 2003).

Therefore, to date, there has been direct evidence for enhanced hippocampal neurogenesis due to PUFA consumption, but only indirect evidence for enhanced neurogenesis in the hypothalamus. Possible links between the BDNF in the regulation of feeding behaviour, and the role of PUFAs in enhancing neurogenesis suggest that this neurotrophin could act as an easily measurable circulating marker of this relationship.

#### 7.1.2.3 Additional Dietary Factors Affecting Neurogenesis

A comparative reduction in energy intake has many health benefits and has been shown to offer protection against neuronal loss *via* increased adult neurogenesis (Levenson & Rich, 2007). It is known that neurogenesis decreases with age; therefore, as PUFAs have been shown to enhance learning during old age (Willis *et al.,* 2009); it may be as a result of increased neurogenesis. Previous work has been carried out comparing different types of dietary supplementation, including high-carbohydrate *vs.* high-fat (Chavez *et al.* 1998), high-fat *vs.* high-sugar (Archer *et al*., 2004) and omega-3 diets (Huang *et al.,* 2004) and studies of neurogenesis (Lindqvist *et al.,* 2006; Hwang *et al.,* 2008; Park *et al.,* 2010; McNay *et al.,* 2012) in several rodent models. However, there is still a need for clarification of the particular neuronal populations affected by dietary changes, and the processes involved.

### 7.1.3 Environmental Enrichment and Neurogenesis

As described previously, in Chapter 6 (Section 6.3.1), the effect of adding a single play tube to each laboratory rat's cage was piloted and shown to increase cell proliferation above baseline levels. However, this result was irreproducible, a fact which did not become apparent until dietary studies, detailed in Chapters 3 and 4, were underway and the concurrent expanded enrichment study had been completed (Chapter 6, Section 6.3.2). Therefore, this simple intervention had been applied before gaining a full appreciation of the likely outcomes and associated limitations. The rationale behind the intervention is detailed in Chapter 6 (Section 6.1.3 and 6.1.4). In brief, given the baseline sparsity of cell proliferation in unstimulated (singly housed, sedentary) Wistar rats previously observed in-house, and supported indirectly by the learning and cognition literature (Kempermann *et al.,* 1997), it was thought that stimulating proliferation to a quantifiable level would provide the cellular density range necessary to allow for comparison of different dietary

interventions; for example, it was anticipated that the high-SFA diet would inhibit proliferation, but this change would be undetectable from an already minimal level. This consideration was particularly important with respect to the hypothalamus, known for its low rate of cell turnover (Koekova *et al.* 2007; Cifuentes *et al.,* 2011).

### 7.1.4 Research Question

***Do dietary PUFAs stimulate hypothalamic neurogenesis?***

#### 7.1.4.1 Aims & Expected Outcomes

To address this question,

1. the quantity of proliferating cells in the hypothalami of PUFA-fed and control rats would be assessed and shown to be increased in the former, and

2. to correlate with altered circulating concentrations of BDNF.

### 7.1.5 Effects of High-Fat Diet on Myogenesis

In addition to dietary factors, exercise is known to improve cognition, learning and memory, and to alleviate depression, possibly through enhancement of neurogenesis in the brain (Hollman *et al.,* 2007; Carek *et al.,* 2011). Intriguingly, there appears to be bidirectional communication between skeletal muscle and neurogenic brain regions, although the identity of these communicating biological factors and underlying mechanisms largely remains to be elucidated (Lista & Sorrentino, 2010). Pharmacological activation of skeletal muscle is suggested to mediate cognitive effects (Kobilo *et al.,* 2011), and so it is reasonable to think that nutraceutical activation could do the same. Dietary PUFAs also stimulate neurogenesis and improve mental health, but how they interact with exercise and skeletal muscle along these parameters also remains to be fully addressed (van Praag, 2009).

The detrimental effects on health of long-term consumption of SFAs are well-established, and as detailed above, include the reduction of cell proliferation within the brain. However, they also impair the ability of individuals to carry out types of endurance exercise, which aid in combating obesity by altering muscle fibre types (Ripple & Hess, 1998; Strømme & Høstmark, 2000). As detailed in Chapter 4, muscles consist of a mixture of different fibre-types (fast and slow), depending on

the function of the whole muscle. However, consuming a diet high in SFAs impairs the ability of muscles to oxidize lipid (Sparks *et al.,* 2005), increasing the tendency to build fast fibres rather than slow fibres, and thereby reducing the capacity for sustained physical activity, and in turn, increasing the chance of weight gain and likelihood of obesity.

In contrast, PUFAs have been shown to protect muscle function, stimulating energy metabolism and increasing the capacity for sustained exercise by improving fatigue resistance (Maillet & Weber, 2006; Demmig-Adams & Carter, 2007) through the reduction of whole-body oxygen demand during extended periods of exercise (Peoples *et al*., 2008). As the consumption of SFAs has been shown to have a detrimental effect on muscle composition and brain neurogenesis, it is a logical step to suggest that they may also affect the process of new muscular tissue growth, which is known as myogenesis (Carlson, 1973).

Myogenesis in the adult results from the proliferation of satellite cells, which lie just under the muscle external lamina (Collins *et al.,* 2005; Zammit *et al.,* 2006). The population is a mixture of stem and precursor cells, and it is the latter which are activated under conditions of muscle regeneration after trauma (Bodine-Fowler, 1994; Ten Broek *et al.,* 2010), or of hypertrophy in response to loading, for example, during exercise (Kadi *et al.,* 2005; Cassano *et al.,* 2009; Smith & Merry, 2012). The extent of muscle repair depends on the viability and extent of the satellite pool, dependent on the presence of certain growth factors in the myogenic niche (Bodine-Fowler, 1994). Proliferation has been shown to be inhibited by ageing (Stoll *et al.,* 2011) and high-fat diet feeding (Kokoeva *et al.,* 2005). For example, it has been shown that postnatal exposure to a diet high in SFAs results in excessive intramuscular fat accumulation, in Sprague-Dawley rats at 12 weeks of age (Yang *et al.,* 2012). This accumulation is associated with a decrease in muscle glycogen and impairment of myogenesis, suggesting that the potential for inappropriate muscle growth contributes to the predisposition for developing a metabolic syndrome-like phenotype during adulthood (Yang *et al.,* 2012). Mice which develop insulin resistance from chronic high-fat feeding have also shown impairment of satellite cell proliferation (Hu *et al.,* 2010); although in this case, this was independent of raised circulating free fatty acids, which indicates that the mechanism for this change is

uncertain. The effect of PUFAs on myogenesis, and whether they can restore proliferation and regeneration, has yet to be investigated.

Markers of DNA synthesis, including the endogenous marker of cell proliferation Proliferating Cell Nuclear Antigen (PCNA) and BrdU, have been used successfully to show proliferation in muscle (Sha *et al.,* 1997; Peterson *et al.,* 2008; Jump *et al.,* 2009; Rathbone *et al.,* 2009). It was thought that examination of BrdU uptake in muscles sampled from diet-fed animals in the current study (Chapter 3) might provide support for any observed alterations in fibre composition or overall numbers; although proliferation would not indicate fate (fibre-type), unless tracked along a time course, it would still indicate developmental activity. BrdU-immunoreactive satellite cells in muscle would also provide an additional indicator, after spleen, of the extent of peripheral uptake, thereby acting as a technical control.

### 7.1.6 Research Question

***Do dietary fatty acids differentially influence the extent of myogenesis in skeletal muscle?***

#### 7.1.6.1 Hypothesis

Changes in skeletal muscle fibre-type populations induced by dietary fatty acids are underpinned by changes in levels of myogenesis.

#### 7.1.6.2 Aim

To test this hypothesis by determining whether high-SFA and –PUFA feeding result in different quantities of proliferating cells in skeletal muscle of rat.

#### 7.1.6.3 Expected Outcomes

Skeletal muscle of PUFA-fed rats would display higher numbers of proliferating cells than that of SFA-fed rats.

## 7.2 Materials & Methods

Details of animal husbandry, maintenance, treatments and tissue processing are described in detail in Chapters 2-6; however, a brief summary is included below. Steps leading to the generation of tissue for analysis of cell proliferation in dietary studies (Chapters 3 and 5) are also summarised below in Fig. 7.1.

### 7.2.1 Animals and Treatment – *Chapters 3&4*

Adult male Wistar rats (~250 g) were stratified by body weight and randomly assigned to one of three groups (*n*=8/group). For eight weeks, they were fed isoenergetic diets, enriched with either SFAs or PUFAs (40% of energy from fat, mainly from lard and fish oil, respectively) or a standard (low-fat) chow (10% of energy from fat, from soybean oil). A summary of the diet compositions can be seen in Table 3.1. Rats received the cell proliferation tracer, bromodeoxyuridine (BrdU) dissolved in their drinking water (1 mg/ml) for the last seven days of the study, at which time a single play tube was introduced to the cage as an enrichment tool. Rats were perfuse-fixed and brains, gastrocnemius and soleus muscle removed for histological analysis of BrdU uptake and other cell proliferation markers, as described in Chapter 2 (Section 2.9). The brain regions examined include the third ventricular lining of the hypothalamus, medial hypothalamus and the dentate gyrus of the hippocampus. Full details of the immunohistochemical procedure can be seen in Chapter 2 sections 2.10.4-7 and Table 2.7.

### 7.2.2 Animals and Treatment – *Chapter 5*

Adult male Wistar rats (~250 g) were stratified by body weight and randomly assigned to one of three groups (*n*=6/group). Two groups were fed isoenergetic diets for three weeks, enriched with either SFAs or PUFAs (40% of energy from fat, mainly from coconut oil and a commercially purified source of the omega-3 fatty acid, docosahexaenoic acid (DHA; Incromega™ Marine Lipid Concentrate E1070, Croda Europe Ltd., Leek, Staffs, UK), respectively) or a standard (low-fat) chow (10% of energy from fat, from soybean oil). A summary of the diet compositions can be seen in Table 5.1. Rats in the original characterisation study (Chapter 3) had received the cell proliferation tracer, BrdU, in their drinking water for the last week of dietary exposure. By the time the study described here was planned, the

limitations of BrdU use were becoming clear. Thus, these animals did not receive BrdU, but were examined for other histological markers of cell proliferation instead. As a potential stimulus for this process, a play tube was placed in each cage for the duration of the study for all groups. Rats were perfuse-fixed and brains removed for histological analyses. The brain regions examined include the third ventricular lining of the hypothalamus, medial hypothalamus and the dentate gyrus of the hippocampus. Full details of the immunohistochemical procedure can be seen in Chapter 2 sections 2.10.4-7 and Table 2.7.

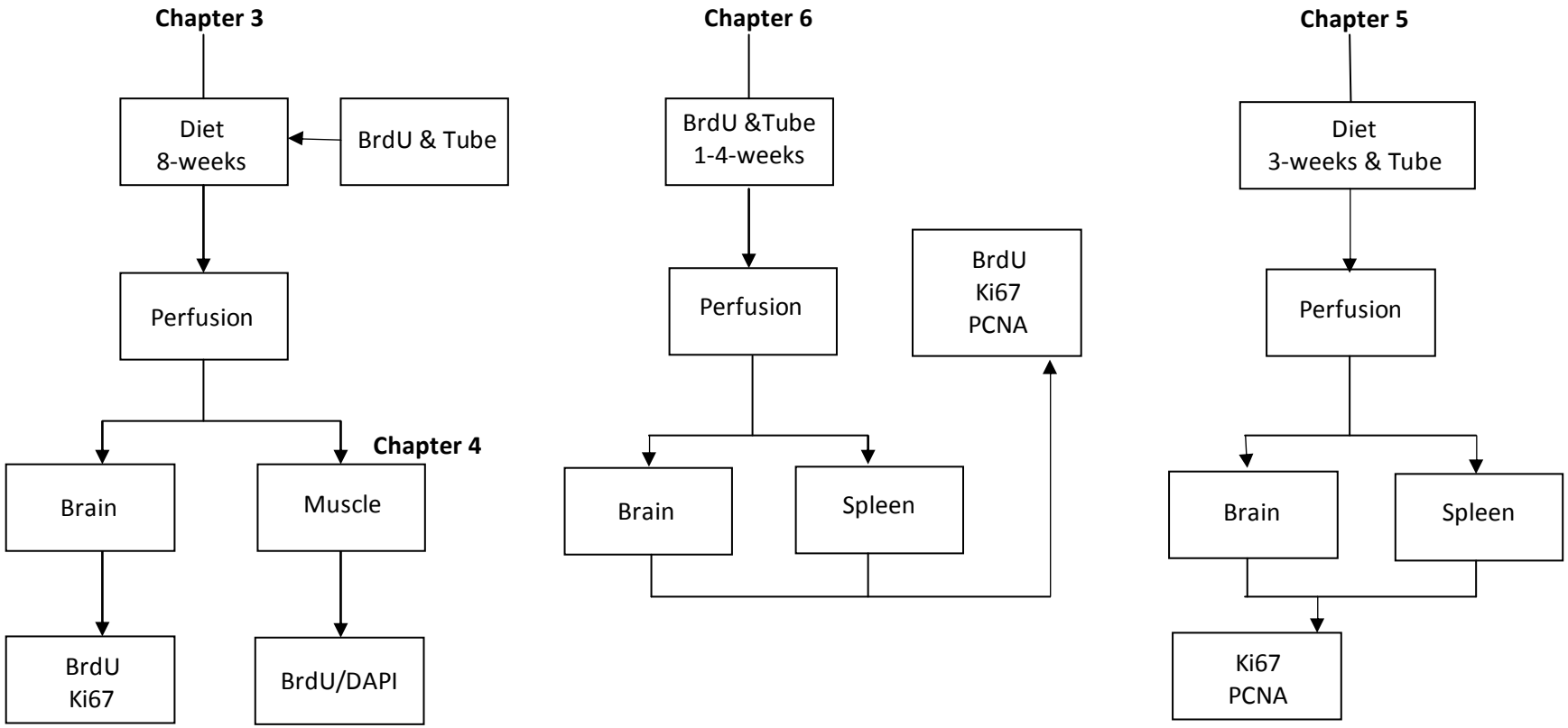


**Figure 7.1.** A summary of steps leading to the generation of tissue for analysis of cell proliferation in dietary studies (Chapters 3 and 5).

## 7.3 Results

### 7.3.1 Effects of High-SFA & High-PUFA Diets on Cell Proliferation

Cell proliferation was largely absent from hypothalamic and hippocampal regions in all diet groups administered BrdU (Figs. 7.2A-F). Positive BrdU immunoreactivity was observed in the roof of the third ventricle (Figs. 7.3A-C) and in the lateral ventricular wall (Figs. 7.3D-F), but this was sparse and occurred to a similar minimal extent in all groups; i.e. irrespective of diet. Similarly, proliferation was extremely scant in gastrocnemius and soleus muscle taken from the same animals, also irrespective of diet (Figs. 7.4A-D). More extensive staining was observed in the *pia mater* (Figs. 7.2G-I), however, verifying BrdU administration and staining technique. However, this did not prove that BrdU had crossed the BBB, and therefore, confirmation was sought that the lack of specific staining reflected a real absence of proliferation. Thus, immunoreactivity against the endogenous proliferation marker, Ki-67, was examined, but also found to be equally scant in all groups (Figs. 7.3B, C, E & F).

### 7.3.2 Effects of High-SFA & High-Omega-3 Diets on Cell Proliferation

Similarly, cell proliferation, as indicated by Ki-67 and PCNA immunoreactivity, was largely absent from hypothalamic and hippocampal regions in all diet groups (Figs. 7.5A-F & 7.6A-F). Positive staining in *pia mater* (Figs. 7.5G-I & 7.6G-I) and spleen (Figs. 7.7C, F) in both cases suggested that scarcity of new cells in regions of interest was real, and not the result of failed technique.

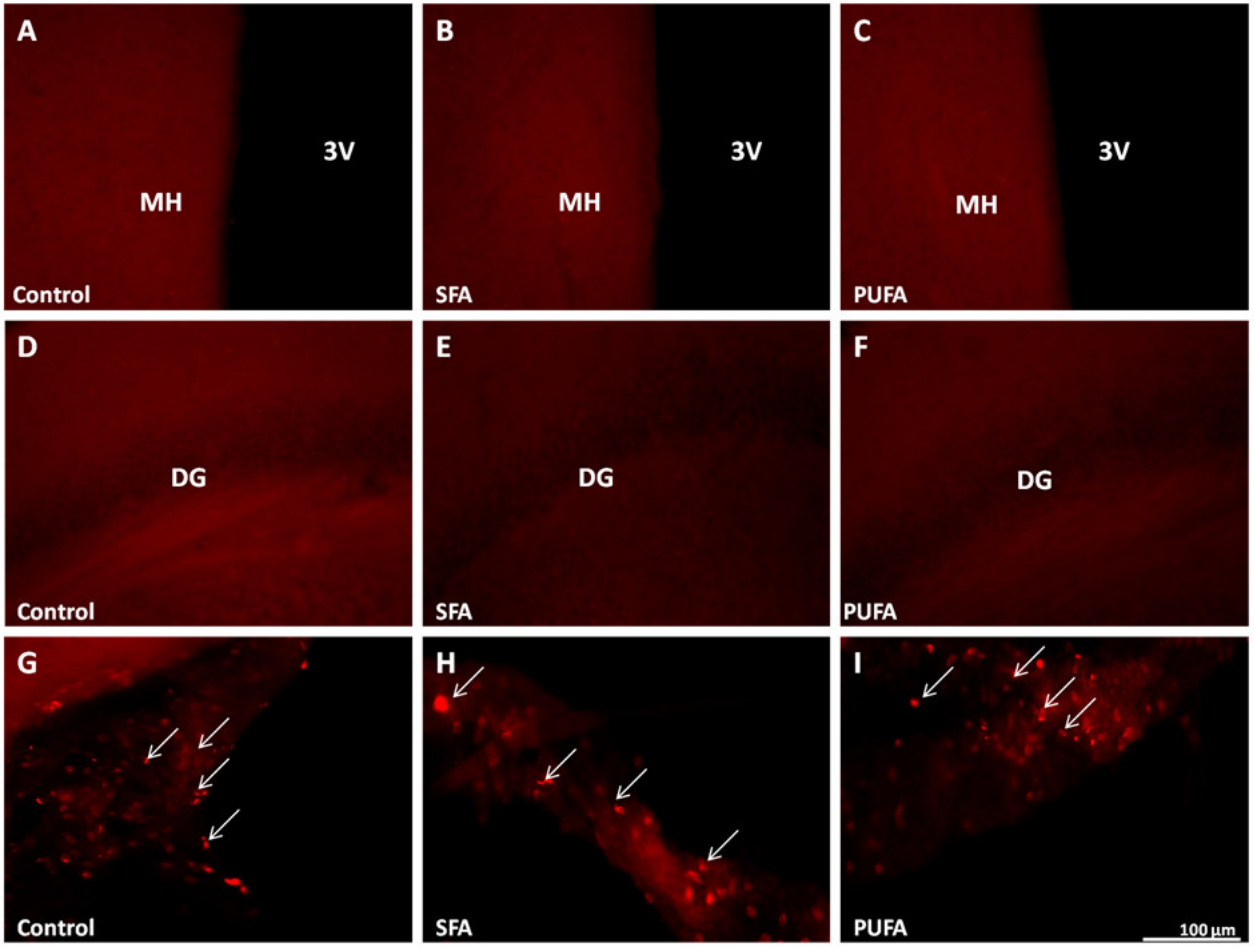


**Figure. 7.2. Chapter 3.** Representative photomicrographs of cell proliferation in response to high-fat feeding. Rats were fed control, high-SFA or high-PUFA diets for eight weeks (*n*=8/group). Proliferating cells show BrdU-positive immunoreactivity in cell nuclei (examples indicated by arrows). **Proliferation was absent in all groups** in the third ventricular (3V) lining and medial hypothalamus (MH; to the left of the 3V; A-C) and in the dentate gyrus (DG) of the hippocampus (positive control; D-F). However, BrdU uptake was confirmed in the *pia mater* (positive control; G-I)

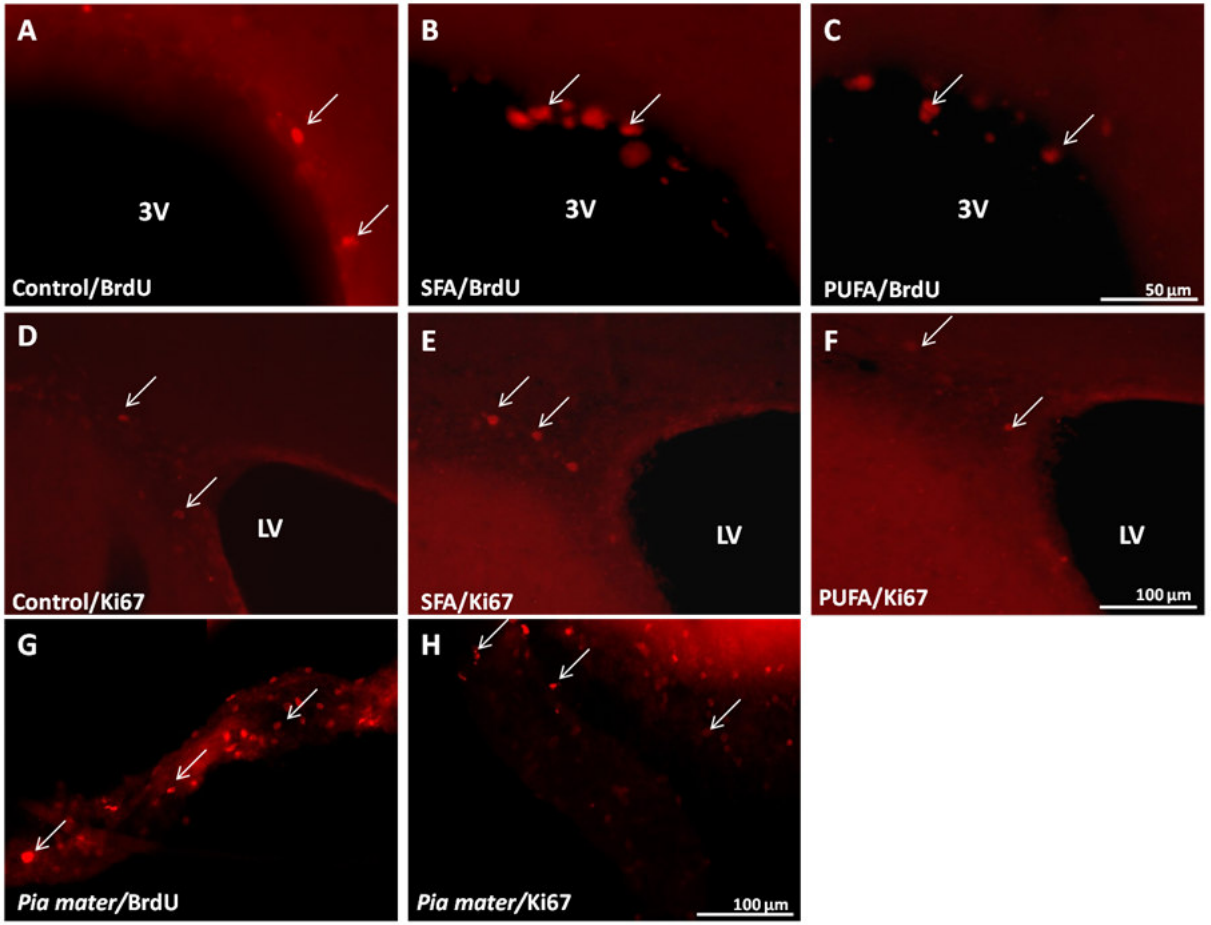


**Figure. 7.3**. **Chapter 3.** Representative photomicrographs of cell proliferation survey in response to high-fat feeding. Rats were fed control, high-SFA or high-PUFA diets for eight weeks (*n*=8/group). Proliferating cells show BrdU- (A-C) and Ki-67-positive immunoreactivity (D-F) in cell nuclei (examples indicated by arrows). **Although sparse proliferation observed as BrdU-positive cells in the roofs of the third and lateral ventricles was confirmed by the endogenous marker Ki-67, neither indicated a differential effect of diet.** Antibody function and staining conditions were confirmed in the *pia mater* (positive control; G, H). **Abbreviations:** 3V = third ventricle; LV = lateral ventricle.

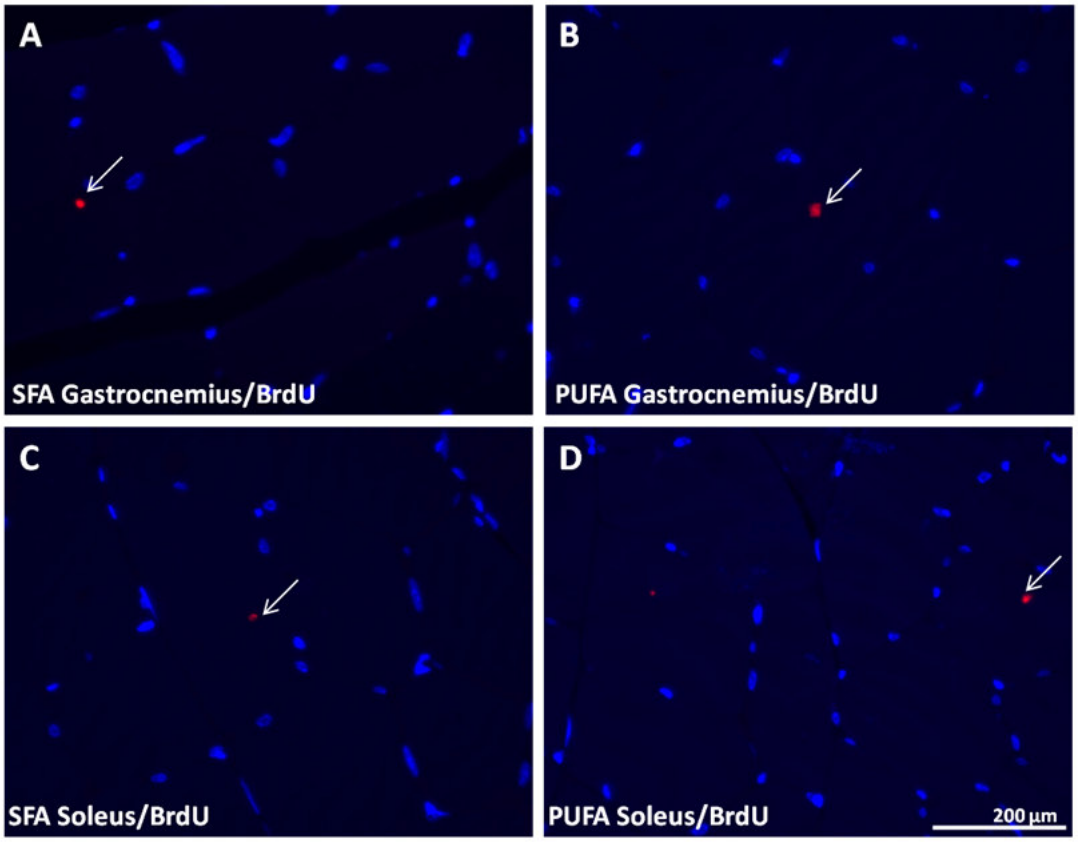


**Figure 7.4. Chapter 4.** Representative photomicrographs of cell proliferation in muscle in response to high-fat feeding. Rats were fed high-SFA or high-PUFA diets for eight weeks (*n*=8/group). *In this instance SFA-fed rats act as a control for PUFA-fed rats.* BrdU-immunoreactive cell nuclei are shown in transverse sections of gastrocnemius (A, B) and soleus muscle (C, D; red fluorescence indicated by arrows). **There was no differentiating effect of diet on proliferation in either muscle type.** Sections were counterstained with DAPI (blue fluorescent nuclei).

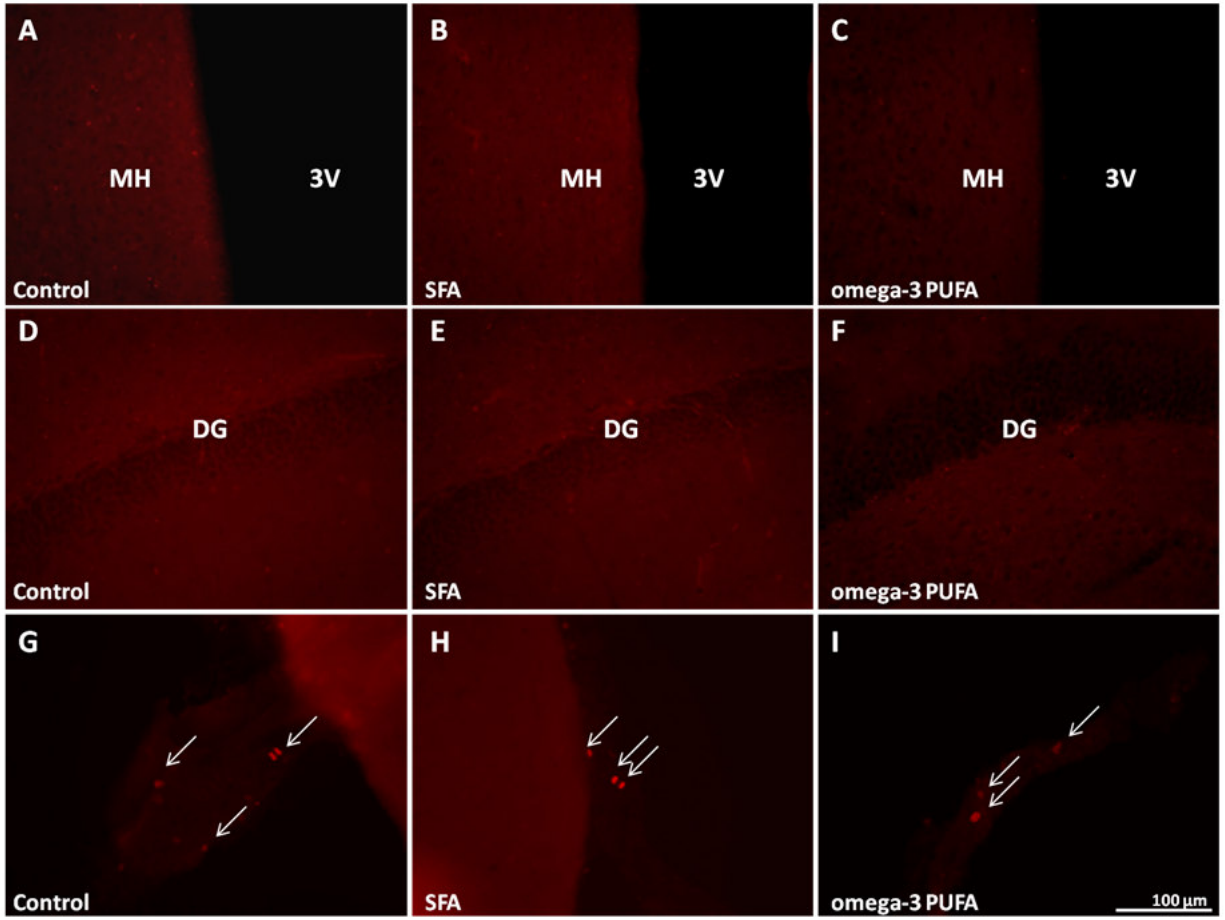


**Figure. 7.5**. **Chapter 5.** Representative photomicrographs of cell proliferation in response to high-fat feeding. Rats were fed control, high-SFA or high-PUFA (omega-3) diets for three weeks (*n*=6/group). Proliferating cells show Ki-67-positive immunoreactivity in cell nuclei (examples indicated by arrows). **Proliferation was absent in all groups** in the third ventricular (3V) lining and medial hypothalamus (MH; to the left of the 3V; A-C), dentate gyrus (DG) of the hippocampus (positive control; D-F). Antibody function and staining conditions were confirmed in the *pia mater* (positive control; G-I). BrdU had not been administered to rats due to lack of confidence in the technique.

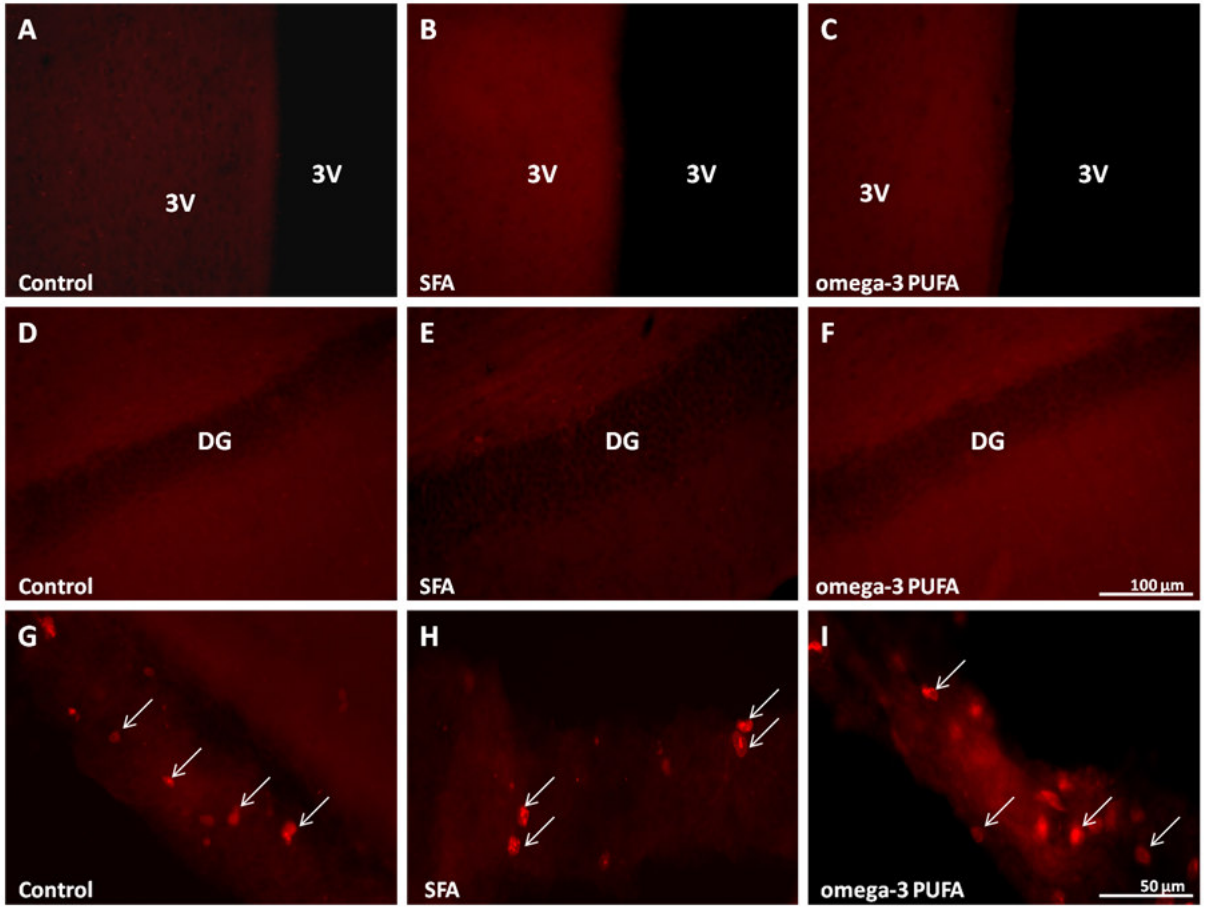


**Figure. 7.6. Chapter 5.** Representative photomicrographs of cell proliferation in response to high-fat feeding. Rats were fed control, high-SFA or high-PUFA (omega-3) diets for three weeks (*n*=6/group). Proliferating cells show PCNA-positive immunoreactivity in cell nuclei (examples indicated by arrows). **Proliferation was absent in all groups** in the third ventricular (3V) lining and medial hypothalamus (MH; to the left of the 3V; A-C), dentate gyrus (DG) of the hippocampus (positive control; D-F). Antibody function and staining conditions were confirmed in the *pia mater* (positive control; G-I). BrdU had not been administered to rats due to lack of confidence in the technique.

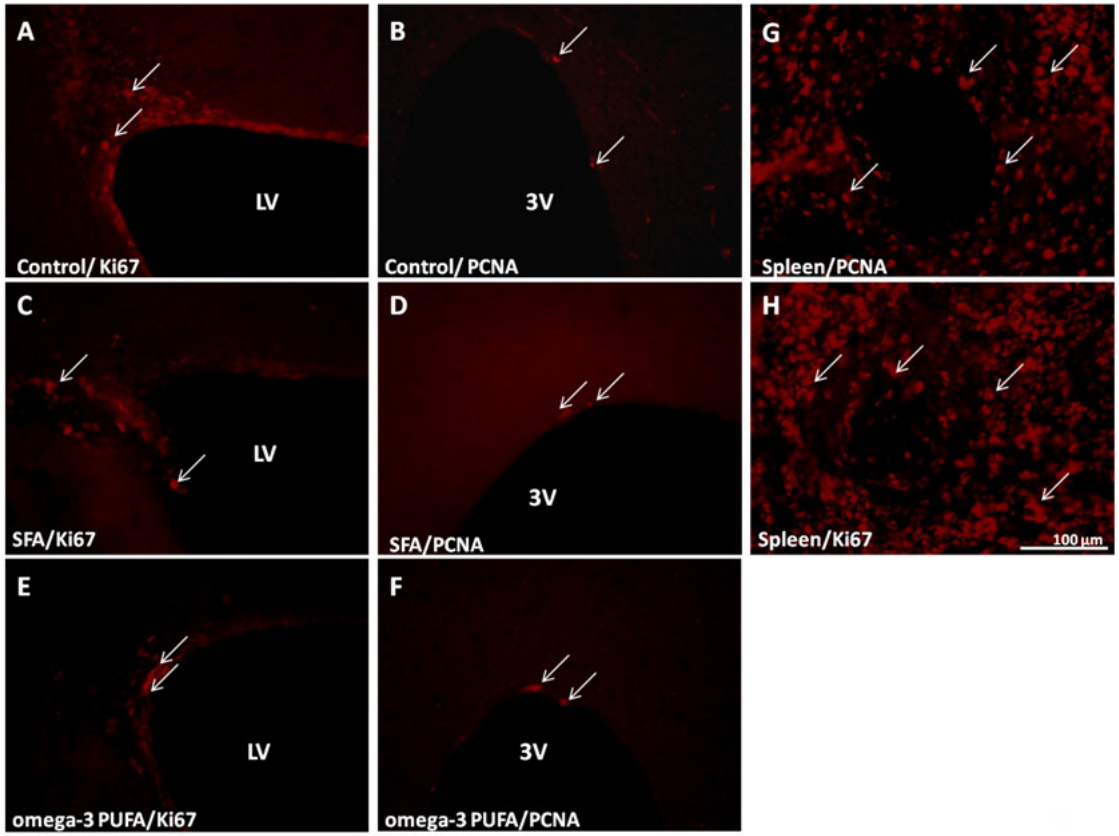


**Figure 7.7. Chapter 5.** Representative photomicrographs of cell proliferation survey in response to high-fat feeding. Rats were fed control, high-SFA or high-PUFA (omega-3) diets for three weeks (*n*=6/group). Proliferating cells show Ki-67- (A, C, E) and PCNA-positive immunoreactivity (B, D, F) in cell nuclei (examples indicated by arrows). **Although sparse proliferation was observed in all groups as PCNA-positive cells in the roof of the 3V, this distribution was not mirrored by Ki-67, observed only in the wall of the LV. Neither endogenous marker indicated a differential effect of diet.** BrdU had not been administered to rats due to a lack of confidence in the technique. Antibody function and staining conditions were confirmed in the spleen (positive control; G, H). Variation in staining distribution reflects the structure of the spleen; i.e. the central region showing fewer stained nuclei is white pulp (as opposed to the surrounding red pulp). **Abbreviations:** 3V = third ventricle; LV = lateral ventricle.

## 7.4 Discussion

### 7.4.1 Effects of Diet Composition on Central Cell Proliferation

All diet groups, high-SFA-, high-PUFA- and low-fat-fed controls, described in Chapter 3, showed similarly absent or minimal levels of cell proliferation in the established neurogenic niches of the hippocampal DG and SVZ of the lateral ventricle, as well as the third ventricular lining and parenchyma of the hypothalamus. BrdU immunoreactivity in the *pia mater* (positive control tissue) suggested that this minimal staining was real, rather than an artefact of suboptimal BrdU administration. However, without being able to compare to the proliferation level in the *pia mater* of rats in the successful pilot enrichment study (Chapter 6), it is impossible to be certain.

On the one hand, the lack of effect by the high-SFA diet may appear to be in contrast to repeated findings that consumption of such diets inhibits cell proliferation and neurogenesis in the rodent DG and hypothalamus (Kokoeva *et al*., 2005; Lindqvist *et al.,* 2006; Park *et al.,* 2010; McNay *et al.,* 2012), but on the other, a near-absence of proliferation in the control animals as well prevents a meaningful comparison. However, it may be that the effects of SFAs on neurogenesis are not entirely predictable. Examination of dietary intervention in neurodegenerative disease has provided some insight into the effects of high-fat diet on neurogenesis. For example, the ketogenic diet, which is used to treat epilepsy, and which is very high in SFAs (80% of energy from fat), also fails to alter the number of BrdU-immunoreactive cells in the DG of Wistar rats from that in low-fat-fed controls (5% of energy from fat) after a month of feeding (Strandberg *et al.,* 2008). The general sparsity of new cells in the current study was similar to that reported in the full (repeat) enrichment study described in Chapter 6, confirming that the play tube was an insufficient stimulus to cell proliferation. Hence, the baseline level of proliferation remained too low to allow for any quantifiable reduction that may have been induced by the high-SFA diet.

It was hypothesized that the high-PUFA diet would enhance hypothalamic neurogenesis. This is supported indirectly by extensive literature (Kawakita *et al*., 2006; Beltz *et al.,* 2007; Ma *et al.,* 2008; Valente *et al*., 2009; Dyall *et al.,* 2010; Fernández-Fernández *et al.,* 2012). However, the lack of effect could be explained by

the fact that the PUFAs were either not at a sufficient concentration to stimulate proliferation above this baseline, or that any stimulatory effect they may have had was overridden by the inhibitory effect of the SFAs also present in the diet (Chapter 3, Section 3.4). SFAs inhibit neurogenesis *via* mechanisms involving neuroinflammation. Rodent models of obesity induced by high-SFA diet feeding are characterised by inflammation both in peripheral tissues and areas of the hypothalamus involved in energy homeostasis (Monje *et al.,* 2003; Molina-Holgado & Molina-Holgado, 2010; Cota & Marsicano, 2011; Thaler *et al.,* 2012). Unlike inflammation in peripheral tissues, however, which develops as a consequence of obesity (Posey *et al*., 2009), hypothalamic inflammatory signaling is evident in rats within three days of high-SFA diet consumption, before the development of weight gain, and markers of neuron injury and inflammation are evident in the arcuate nucleus within one week (Thaler *et al.,* 2012). Although these responses temporarily subside, suggesting that neuroprotective mechanisms may initially limit the damage, with continued high-SFA diet feeding, inflammation returns permanently and impairs neurogenesis (Monje *et al.,* 2003; Molina-Holgado & Molina-Holgado, 2010; Cota & Marsicano, 2011; Thaler *et al.,* 2012). Although PUFAs are neuroprotective and anti-inflammatory (Dyall, 2010; Hashimoto & Hossain, 2011), they may not have been at a sufficient concentration in the high-PUFA diet to prevent or repair damage caused by the SFAs also present. This suggests that PUFAs, rather than directly stimulating neurogenesis, would act to protect normal levels of proliferation.

Again, however, other research indirectly suggests that the current findings may not be surprising. Rats fed for three months on diets as high in fat as those used here (40% of energy from fat) displayed equal impairments in various learning paradigms, regardless of the fat source [lard (SFAs) or soybean oil (PUFAs)] (Winocur & Greenwood, 1993; 2005). The effect was concentration-dependent in rats fed high-SFA diets (20% *vs.* 40%), but specific to the highest level of PUFAs (Winocur & Greenwood, 2005). Based on the association between hippocampal neurogenesis and cognition, learning and memory (Amrein & Lipp, 2009; Noble *et al.,* 2011), these findings indirectly support those here. The mechanisms behind these impairments were not investigated by the authors, although they did consider the involvement of diet-induced insulin resistance in reducing glucose uptake by the

brain. This is thought to contribute to the cognitive decline seen both with ageing and type 2 diabetes (Gage *et al.,* 1984; Cunnane *et al.,* 2011; McNay & Recknagel, 2011). Conversely, Winocur and Greenwood (2005) showed that i.p. glucose treatment improved memory function in the high-fat-fed rats used in the various learning paradigms by enabling glucose metabolism in the hippocampus. Therefore, it is reasonable to suggest that the reversal of glucose intolerance may in turn reverse the inhibition of neurogenesis by dietary fats. This idea is further supported by evidence that memory dysfunction in aged animals, a process linked to a decline in cell proliferation, correlated with an impairment in glucose metabolism (Gage *et al.,* 1984). Furthermore, genes involved in the breakdown of glucose (Glucose-6-phosphate dehydrogenase) are upregulated during differentiation of Neural Stem Cells (NSC) (Gurok *et al.,* 2004; Rafalski & Brunet, 2011). Glucose-6-phosphate dehydrogenase is an important factor for cell growth. Studies have shown that the inhibition of this enzymes activity prevents the incorporation of [3H]thymidine incorporation into cells, but, over expression stimulated cell growth, as measured by an increase in [3H]thymidine incorporation (Tian *et al.,* 1998). The likely insulin resistance observed in all three groups in the current study, reflected in their hyperinsulinaemia (Chapter 3, Fig. 3.9), may have impaired central glucose uptake, thereby impairing cell proliferation and accounting for the low levels seen here.

### 7.4.2 Effects of High-Fat Diets on Myogenesis

The sparse cell proliferation observed in skeletal muscles from both high-SFA- and high-PUFA-fed rats is consistent with that observed centrally, although the possible issue of whether this is an artefact of the administration method still remains (Chapter 2, Section 2.9.5). If the findings are real, however, the equivalent satellite cell proliferation in the two high-fat-fed groups supports their similarities in muscle mass (Chapter 3, Fig. 3.3D), muscle fibre proportions and numbers of fibres overall (Chapter 4, Section 4.3 and Fig. 4.3). The lack of satellite cell activation indicates that no significant muscle regeneration or hypertrophy had been taking place. This makes sense, as new muscle cell growth is most common following injury (Zammit *et al.* 2006; Kuang *et al.,* 2008; Cassano *et al.* 2010), but here there had been no trauma here; nor had there been significant hypertrophy due to loading (the sedentary cage environment precludes exercise). This raises the question of whether diet alone is an intervention capable of stimulating muscle regeneration. Indeed, originally it

was thought that once a muscle had formed, the body was no longer capable of producing any new muscle fibres, should damage occur. However, it is now known that the fibre-type ratio of soleus muscle can be altered to one of increased Type I oxidative fibres, and can undergo regeneration, in rats fed the dietary supplement, acetyl-L-carnitine (Cassano *et al.,* 2010). Although the mechanisms behind these types of changes have yet to be determined (Howald *et al.* 1985; Bodine-Fowler, 1994; Zammit *et al.* 2006; Kuang *et al.,* 2008; Cassano *et al.* 2010), satellite cells are thought to be involved in this process of alteration in muscle composition (Zammit *et al.* 2006; Kuang *et al.,* 2008; Cassano *et al.* 2010). In addition to the proliferative action of amino acids, the published findings reviewed in Chapter 4 and above, in Section 7.1.5, indicate that dietary FAs are also able to alter muscle composition and that SFAs inhibit proliferation in muscle, as they do in brain (Hu *et al.,* 2010; Yang *et al.,* 2012). Whether PUFAs counteract this inhibition and/or stimulate proliferation in muscle, as they have been shown to do in brain (Kawakita *et al*., 2006; Beltz *et al.,* 2007; Ma *et al.,* 2008; Dyall *et al.,* 2010), still remains to be seen. Again, it is reasonable to suggest that the presence of SFAs in the high-PUFA diet used in the current study would have overridden any proliferative effect they may have had (Chapter 3, Section 3.4). Finally, had immunohistochemical staining been carried out for markers of satellite cells, such as Pax7, MyoD and lysenin, positive immunoreactivity may have indicated the stage in myogenesis at which the satellite cells were (Nagata *et al.,* 2006; Zammitt *et al.,* 2006), possibly indicating that the muscle was undergoing change that had not yet manifested itself as a change in fibre-type *per se*.

### 7.4.3 Effects of Diet High in Omega-3 FAs on Central Cell Proliferation

Intervention with a purified dietary source of PUFAs also failed to stimulate substantial cell proliferation, either in the hypothalamus or hippocampus. This is in contrast to a number of previous findings showing enhanced neurogenesis in animals fed diets enriched with DHA, the omega-3 FA used here (Kawakita *et al*., 2006; Beltz *et al.,* 2007; Ma *et al.,* 2008; Dyall *et al.,* 2010). Aside from the possible reasons discussed above and in Chapter 6 (Section 6.4), including issues of enrichment and rat strain, this lack of effect is consistent with similar circulating BDNF concentrations across all the diet groups for the duration of the study. However, there is a tantalising hint, from the acute, albeit non-significant, increase in

BDNF concentration at three weeks of high-omega-3 exposure, that had the study run for longer, BDNF levels may have become sufficient to stimulate proliferation. Omega-3 FA consumption has been shown to stimulate secretion of BDNF after one and ten months (Wu *et al.,* 2004; Bousquet *et al.,* 2009), both time periods longer than the one studied here. As an increase in BDNF concentrations is known to indirectly stimulate neurogenesis by increasing synaptic transmission and long-term potentiation (Gomez-Pinilla, 2011), this may then have led to an increase in observed cell proliferation also.

Although the link between dietary DHA supplementation and hippocampal neurogenesis has been proven *in vivo* (Kawakita *et al.,* 2006), it is possible that it had the opposite effect here, on cell proliferation *per se*. There are two possible reasons for this. First of all, DHA has been shown to stimulate neurogenesis by controlling the transcription factors responsible for regulating the cell cycle (Ohnuma *et al.,* 2001; Cremisi *et al.,* 2003). Specifically, it inhibits cell proliferation but promotes neuronal differentiation by promoting cell cycle exit and suppressing cell death (Kageyama *et al.,* 2005; Kawakita *et al.,* 2006; Kageyama *et al.,* 2008). In addition, DHA's effects are concentration-dependent, whereby high concentrations reduce survival by inducing apoptosis leading to neuronal death (Kim *et al.,* 2000; Katakura *et al.,* 2009). This suggests that the concentration of DHA used in the current study may have been so high as to have the opposite effect to that desired, inhibiting, rather than stimulating, cell proliferation, and accounting for the low levels of proliferation observed. Although this effect had been discovered *in vitro*, in cultured neural stem cells, and therefore, cannot be directly translated to the *in vivo* state, a similar principle may apply. The equivalent concentrations of diet-incorporated DHA and their *in vivo* effects will have to be determined. Humans taking supplements can safely consume 120 mg per day of DHA for general health purposes, but this can be increased to 1.4 g per day to control specific medical conditions, such as high cholesterol and blood pressure, by the lowering of circulating triglycerides (Zargar & Ito, 2011). The concentration of DHA within the diet used here was quite high: Weight per weight, rats consumed, on average, 26 grams of food per day, 20% of which was fat. Of this, 96% was provided by omega-3 FAs, and in turn, 82% of this by DHA; therefore, approximately 4 grams of DHA were consumed per day. This dose is almost three times greater than that recommended for therapeutic purposes to

the human population, and effectively even greater, when the small body size of the rat is considered. However, whether this is an issue depends on whether DHA is metabolised in the same way in rats and humans.

## 7.5 Conclusion

The studies described here aimed to investigate whether PUFA consumption would stimulate hypothalamic neurogenesis and alter myogenesis in skeletal muscle. It is possible that neither effect was seen because the diet composition was not conducive to cell proliferation, due to the presence of inhibitory SFAs (Chapter 3). However, similar minimal levels of proliferation in response to a purified PUFA source (Chapter 5), as well as to control diets, suggest that other influences on neurogenesis in the current model must be disentangled and identified before the effects of dietary FA intervention can be determined.

# CHAPTER 8

## GENERAL DISCUSSION AND FUTURE PERSPECTIVES

# General Discussion and Future Perspectives

## 8.1 Summary

Feeding the male Wistar rat for chronic (3-8-week) periods with isoenergetic diets highly enriched with saturated and polyunsaturated fatty acids (SFAs and PUFAs) provided valuable insight into the differential effects of these FA classes on body weight regulation. The primary aim of this project had been to create a rational model of chronic PUFA consumption for future application in studies of nutraceutical treatment of obesity. On the whole, this was achieved, and the design requirement for comparison with an equally high-SFA-fed model expanded our current knowledge of the character of diet-induced obesity. Although the mechanisms by which PUFAs exert long-term body weight control remain to be fully elucidated, the experiments described in this thesis have revealed that they do so, at least in part, through improved feeding behaviour and body composition.

The secondary aim of the project had been to develop methods for stimulating and visualising neurogenesis in the hypothalamus of rat, in ways compatible with dietary manipulation, given the growing body of evidence for this region as a nutrient-responsive neurogenic niche. It was hoped that if this could also be carried out successfully, the conditions would be set for the future combining of the two sets of interventions, dietary and neurogenic, to determine whether stimulation of neurogenesis is one of the means by which PUFAs regulate body weight. This proved more problematic, such that this second aim could not be addressed. However, the technical difficulties incurred did generate the research questions which must be answered next to progress toward this aim. The following discussion explores what might be done to address these remaining gaps in the knowledge and continue to advance the field.

## 8.2 Salient Findings and Future Work

### 8.2.1 Dietary Studies

Previous work on human body weight metabolism had examined only the effects of short-term consumption of PUFA supplements (Lawton *et al.*, 2000; Buckley & Howe, 2009). Attempts to model in rodents long-term consumption of PUFAs

incorporated into chow have been difficult to interpret because of a general lack of isoenergetic controls (Ricci & Ulman, 2008). The studies described here were designed to address these shortcomings.

Rather than simply repeat previous work, studies were expanded to examine in closer detail the circadian rhythmicity of PUFA feeding, and for the first time have revealed that the satiety with which it has previously been associated (Scharrer, 1999; Lawton *et al.,* 2000) occurs during the day (Chapter 3). Importantly, however, this exciting effect is transient, showing acute enhancement after three weeks of consumption, but gradually disappearing after that. Surprisingly, this enhancement was observed with a PUFA sourced from menhaden fish oil, which also contains SFAs, but not with a purified PUFA, the omega-3 FA, DHA (Chapter 5). Satiating effects of DHA (Ogawa *et al.,* 2008; Parra *et al.,* 2008) may occur in a different window entirely. Alternatively, the SFAs in the high-fish oil diet may have still been exerting satiating effects at this early stage. Fat-sensing in the gut suppresses ongoing feeding acutely, a mechanism which is eventually lost with chronic SFA feeding, as obesity develops (Buettner *et al.,* 2006). Although the same general pattern was observed in the high-SFA-fed group, it may have fallen short of significance because the higher SFA content of the diet disabled the mechanism sooner. This could be tested by examining concurrent alterations in circadian rhythm and weekly expression of satiety peptides in the gut, such as cholecystokinin (CCK), peptide YY (PYY) and glucagon-like peptide-1 (GLP-1). In diet-induced obesity (DIO), these are reduced, and levels of FA receptors, GPR40 and GPR120 are altered (Duca *et al.,* 2012). PUFAs, on the other hand, are found to be more satiating than SFAs when infused into the upper small intestine in human subjects (French *et al.* 2000) and when incorporated into food items (Lawton *et al.* 2000). These findings may relate to the chemical responses evoked following ingestion of these FAs. CCK has been shown to be released in larger quantities following the consumption of foods containing PUFAs, compared with other types of FAs (Beardshall *et al*. 1989).

The clear improvements in adiposity and lipid metabolism observed with the high-DHA diet encourage the view that this is a valid model of high-PUFA consumption, demonstrating changes to energy metabolism expected from both the human and animal literature (Ruzickova *et al*., 2004; Buckley & Howe, 2009). However, it requires further development to corroborate these findings. For example,

measurement of circulating adipokine and insulin concentrations is now needed to help clarify the apparently opposing relationships between energy intake and adiposity which suggest enhanced energy expenditure. It is expected that the reduced adiposity would be reflected in increased adiponectin levels but reduced insulin and leptin levels, although the increased energy intake contradicts this. Ideally, enhanced energy expenditure would be confirmed through calorimetry testing, for example, and at the molecular level, through demonstration of increased expression of UCP1 in BAT and UCP3 in skeletal muscle (McClave & Snider, 1992; Tsuboyama-Kasaoka & Ezaki, 2001; Mailloux *et al.,* 2012). Indeed, omega-3 FAs are known to alter gene expression involved in increasing lean muscle tissue, fat oxidation and energy expenditure in muscle and decreasing fat deposition in this tissue, but more long-term trials are needed to determine mechanisms of action (Bordoni *et al.,* 2006; Demmig-Adams & Carter, 2007; Izumiya *et al.,* 2008; Shortreed *et al.,* 2009). Histological examination of the muscle from this study (Chapter 5) would hopefully confirm reduced ectopic fat deposition in a long-term design, and would address the aim of Chapter 4, possibly showing a Type I fibre-type profile.

That there were few phenotypic differences between the SFA- and low-fat-fed control groups in Chapter 5 deserves further comment. This suggested that coconut oil, though a purer source of SFAs than lard, was not an appropriate choice for inducing obesity (99% *vs.* 35% SFA content w/w; see Appendices II & IV, where lard contains comparable quantities of SFAs and MUFAs). It has, in fact, been observed previously that lard is the more effective of the two in this respect (Buettner *et al.,* 2006). Closer study of the nutrient breakdown of the diets used here confirms this: coconut oil is comprised of small-medium-chain FAs [lauric (C12) and myristic acids (C14); see Appendix IV], which are more readily oxidised than the longer-chain FAs found in lard [Chapter 3; palmitic (C16), stearic (C18) and oleic acids (C18); see Appendix II], and are, therefore, less likely to be stored in adipose tissue (Papamandjaris *et al.,* 1998; Buettner *et al.,* 2006). Nonetheless, diets utilising coconut oil have been shown to induce obesity following three or more months of feeding, but these diets commonly contain 58-60% of energy as fat (more than the 40% used here; Buchner *et al.,* 2008; Lee *et al.,* 2008). The influence of these FAs (type and concentration) would have to be tested in isolation and across a time course

to determine which might act as the optimal isocaloric control for the PUFA-fed group.

Ideally, an extended time course of diet exposure, including periodic body composition analysis and meal pattern analysis would build on this foundation to more fully characterise the model. This is important, as the transitory nature of enhanced satiety with fish oil could have important implications for human dietary recommendations, as well as the cost implications of purifying omega-3 FAs for incorporation into human foodstuffs.

Further validation of the model should also entail reversal studies, which would confirm the role of PUFAs in improving phenotype; i.e. it would be expected that reduced body weight, adiposity and TG levels would be normalised upon withdrawal of the diet and replacement with the control diet. By using a reversal paradigm, strength is added to the notion that observed changes are indeed due to the dietary intervention.

Finally, a thorough characterization of the model would require investigation of central mechanisms (other than neurogenesis, discussed below). For example, dopaminergic activity could be measured in the LHA and VMH, where it regulates meal size and meal number (IMI), respectively (Meguid *et al.,* 1996; Fetissov *et al.,* 2002), to see if it would correlate with alterations in feeding periodicity in response to the different FAs and their concentrations. Indeed, high-SFA, though not high-PUFA, diets have been shown to activate expected regions of the hypothalamus in mouse (LHA and PVN), but the populations to which these activated neurons belong have not been identified (Wang *et al.,* 1999). Early gene activation in brain tissue harvested from animals studied here was similarly examined by c-Fos immunohistochemistry, but was found to be minimal in all diet groups (data not shown). The reasons for this are unknown at present, but may be related to diet being too subtle a stimulus in the chosen model, as discussed with respect to neurogenesis.

### 8.2.2 Cell Proliferation Studies

Pilot work (Chapter 6) carried out concurrently with the first high-PUFA study (Chapter 3) showed that hypothalamic cell proliferation could be stimulated dramatically in response to simple environmental enrichment in the form of a plastic

play tube introduced to the cage. This non-edible toy supported feeding study requirements, where individual consumption is measured, and so stimulation by social enrichment, for example, from cage companions (Kempermann *et al.,* 1997, 2002; Mak *et al.,* 2007), is not an option. However, these findings could not be reproduced; reinforcing the knowledge that neurogenesis is subject to many influences, including age, species, strain and stress (Kempermann *et al.,* 1997; Lindqvist *et al.,* 2006; Mak *et al.,* 2007; Rafalski & Brunet, 2011), the degrees of influence of which would have to be determined in a series of systematically controlled studies. Failure to stimulate cell proliferation above control levels in DHA-fed animals (Chapter 7) suggested further that change in dietary FA composition is not a powerful enough intervention to stimulate neurogenesis, when used alone in Wistar rat.

Levels of cell proliferation in control animals (low-fat-fed) were absent-to-minimal (irrespective of niche), and so were those in normally active neurogenic niches, such as the DG of the hippocampus and the SGZ of the lateral ventricle (irrespective of diet group), not only when examined with the tracer, BrdU, but also with cell cycle markers, Ki-67 and PCNA. Taken together, these indicate that the focus of attention in future studies can be diverted from issues surrounding tracer administration method, and must fall on creating a model in which barriers to proliferation are eliminated, such that the neuroprotective actions of PUFAs can be enabled. These barriers include impoverished environment, older age, strain (anxious/stressed temperament), and central inflammation. The first two of these have already been discussed (Chapters 6 & 7), but the last two will be expanded upon here.

It seems reasonable to think that a rat strain with a low-anxiety temperament, such as the hooded Long-Evans (LE), which is also more responsive in learning paradigms than albino rats (Harker & Wishaw, 2002; Simpson & Kelly, 2011), might prove to have a naturally high level of neurogenesis. This appears to be the case with juvenile LE rats, which show a similar baseline level of hippocampal cell proliferation and survival of immature neurons to that seen in juvenile wild rats, and which is increased above that of Sprague-Dawley rats. However, this difference disappears in adulthood, as wild and LE levels fall into the normal range for other captive-bred strains (Epp *et al.,* 2009). However, the very fact that a baseline range

could be measured at all in alternative captive strains suggests that, for whatever reason, the Wistar used here is an inappropriate choice for studies of neurogenesis. As this contradicts a number of other studies in which Wistars have been used successfully (e.g. Urbach *et al.,* 2008; Soumier *et al.,* 2009; Zhuang *et al.,* 2012), it points to issues of genetic drift (Kacew & Festing, 1996), discussed in Chapter 3, and as yet unidentified inhibiting factors specific to husbandry conditions applied in this project.

A synthesis of the current obesity and neurodegenerative literature reveals a number of key facts (Yon *et al.,* in submission). These are that

(1) raised circulating concentrations of TGs and pro-inflammatory cytokines, such as TNF-α and IL-6, compromise the integrity of the blood-brain barrier (BBB), blocking access to the brain by neuroprotective factors, such as leptin and insulin. Together with central inflammation, they are the likely cause of impaired central neurogenesis induced by high-SFA feeding (Lindqvist *et al.,* 2006; Stranahan *et al.,* 2008; Thaler *et al.,* 2012). PUFAs are likely to restore neurogenesis through their well-established suppression of TGs and inflammatory cytokines, as well as known improvements to BBB function (Sinn & Howe, 2008; Kuo *et al.,* 2010; Shearer *et al.,* 2012).

(2) PUFAs are thought to stimulate hippocampal neurogenesis through mechanisms involving a number of receptors, including the FA receptor, GPR40, glutamate receptors and retinoic acid receptors (RARs) (de Urquiza *et al.,* 2000; Katakura *et al.,* 2009; Oh & Lagakos, 2011), and to alleviate SFA-induced hypothalamic inflammation *via* activation of GPR120 (Cintra *et al.,* 2012).

(3) SFA-induced impairment of neurogenesis is associated with reduced gene expression and protein levels of BDNF in the hippocampus (Obrietan *et al.,* 2002; Park *et al*., 2010). As PUFAs enhance synthesis, secretion and intracellular signalling of BDNF, they may restore neurogenesis by these means (Wu *et al.,* 2004; Balanzá-Martínez *et al.,* 2011).

These points raise questions as to whether the Wistar rat has inherently low levels of neurogenesis due to strain-specific defects which disable the action of

PUFAs; i.e. a susceptibility to inflammation, a weakened BBB, or innately low levels of key receptors and BDNF. Although information on RARs, RXRs and GPRs is lacking in this area, and it appears that there are no strain-specific differences in levels of glutamate receptors or BDNF expression, it does appear that there is a strain dependence in rodent susceptibility to some inflammatory conditions, and glutamate modulation of activation of other receptor-types (Lariviere *et al.,* 2006; Mori *et al.,* 2010; Mustafi *et al.,* 2012). Although Wistar rats have not been examined in any of these comparisons, the potential for a strain difference is suggested. BDNF gene expression in spinal cord has also been shown to correlate with rat strain differences in neuropathic pain and in hippocampus, with prenatal stress (Herradon *et al.,* 2007; Neeley *et al.,* 2011). Interestingly, the correlation between hippocampal BDNF mRNA expression and general and locomotor activity also differs according to mouse strain (Nesher *et al.,* 2012). This highlights again the important role of exercise as an intermediary between BDNF and neurogenesis (Wu *et al.,* 2008), and suggests that lack of exercise in the current model could be partly responsible for the low neurogenic levels and unchanging BDNF concentrations observed in control animals.

In addition to the extrinsic factors mentioned above, any future model would also have to be tested for the presence of intrinsic factors (local signals and cellular machinery) required for maintenance of the neurogenic niche (Massirer *et al.,* 2011). For example, studies similar to those of Katakura *et al.* (2009) would have to be carried out on cultured hypothalamic cells to see whether the same bHLH transcription factors are at play in response to omega-3 FAs. Indeed, *in vitro* studies would allow for the study of individual neurogenic influences in isolation, which is perhaps a good starting point in an area as complex as the impact of diet on neurogenesis. Adult-derived rat hypothalamic cell lines are now commercially available from the researchers who originally showed that leptin and GLP-1 stimulate neurogenesis in these cells and in the latter case, that this is mediated by CNTF (Belsham *et al.,* 2004, 2009; Dalvi *et al.,* 2011; Cellutions package insert). Substances such as BrdU, individual FAs of different classes and concentrations, and trophic factors could be added to the culture medium and effects on proliferation observed using immunohistochemical techniques.

Although, as mentioned above, stimulation, rather than identification, of proliferating cells has been the key issue here, once a model of optimal neurogenic capacity were created, it might be possible to develop a transgenic rat using similar reporter line technology to that used by Encinas and Enikolopov (2008). They created nestin-CFPnuc mice, in which (hippocampal) neural progenitor cells express fluorescent proteins. On the genetic background of a rat strain with inherently increased neurogenic capacity, this would at least eliminate issues of BrdU administration. As argued above, this would not necessarily address the issue of inherently reduced mitotic capability of hypothalamic cells; i.e. it would not necessarily follow that because high levels of neurogenesis occur in the hippocampus, they would also occur in the hypothalamus. This might still require an extrinsic stimulus, such as i.c.v. infusion of a trophic factor; e.g. BDNF, CNTF, EGF or IGF-1 (Yoshimizu & Chaki, 2004; Kokoeva *et al.,* 2005; Lee & Son, 2009; Noble *et al.,* 2011).

## 8.3 Conclusion

In conclusion, application of appropriate controls for dietary energy content and composition show that benefits to body weight metabolism of long-term consumption of diets highly enriched with PUFAs, and in particular, omega-3 fatty acids can be successfully modelled in rat. However, further work is required to determine the precise timeline of their emergence and underlying mechanisms.

# APPENDICES

# List of Appendices

# Appendix I - Complete nutritional breakdown for diets used in Chapter 3

## Rodent Diets with 10 kcal% Fat (from Lard and Soybean Oil) or 40 kcal% Fat from (mostly) Lard or Menhaden Oil

| | 10 kcal% Fat | | 40 kcal% Fat (Lard) | | 40 kcal% Fat | |
|---|---|---|---|---|---|---|
| **Product #** | **D11100701** | | **D09121201** | | **D09121202** | |
| | gm% | *kcal%* | gm% | *kcal%* | gm% | *kcal%* |
| Protein | 19.2 | *20* | 22.9 | *20* | 22.9 | *20* |
| Carbohydrate | 67.3 | *70* | 45.7 | *40* | 45.7 | *40* |
| Fat | 4.3 | *10* | 20.4 | *40* | 20.4 | *40* |
| Total | | *100.0* | | *100* | | *100* |
| kcal/gm | 3.85 | | 4.58 | | 4.58 | |
| | | | | | | |
| **Ingredient** | **gm** | ***kcal*** | **gm** | ***kcal*** | **gm** | ***kcal*** |
| Casein, 80 Mesh | 200 | *800* | 200 | *800* | 200 | *800* |
| L-Cystine | 3 | *12* | 3 | *12* | 3 | *12* |
| | | | | | | |
| Corn Starch | 510 | *2040* | 205 | *820* | 205 | *820* |
| Maltodextrin 10 | 90 | *360* | 90 | *360* | 90 | *360* |
| Sucrose | 100 | *400* | 100 | *400* | 100 | *400* |
| | | | | | | |
| Cellulose, BW200 | 50 | *0* | 50 | *0* | 50 | *0* |
| | | | | | | |
| Soybean Oil | 25 | *225* | 25 | *225* | 25 | *225* |
| Lard | 20 | *180* | 155.5 | *1400* | 0 | *0* |
| Menhaden Fish Oil (tBHQ at 200 ppm) | 0 | *0* | 0 | *0* | 155.5 | *1400* |
| | | | | | | |
| tBHQ* | 0.038 | *0* | 0.031 | *0* | 0 | *0* |
| | | | | | | |
| Mineral Mix, S10026 | 10 | *0* | 10 | *0* | 10 | *0* |
| DiCalcium Phosphate | 13 | *0* | 13 | *0* | 13 | *0* |
| Calcium Carbonate | 5.5 | *0* | 5.5 | *0* | 5.5 | *0* |
| Potassium Citrate, 1 H2O | 16.5 | *0* | 16.5 | *0* | 16.5 | *0* |
| | | | | | | |
| Vitamin Mix, V10001 | 10 | *40* | 10 | *40* | 10 | *40* |
| Choline Bitartrate | 2 | *0* | 2 | *0* | 2 | *0* |
| | | | | | | |
| FD&C Yellow Dye #5 | 0.025 | *0* | 0.05 | *0* | 0 | *0* |
| FD&C Red Dye #40 | 0.025 | *0* | 0 | *0* | 0.05 | *0* |
| FD&C Blue Dye #1 | 0 | *0* | 0 | *0* | 0 | *0* |
| | | | | | | |
| **Total** | **1055.088** | ***4057*** | **885.58** | ***4057*** | **885.55** | ***4057*** |
| | | | | | | |
| **tBHQ, % of diet** | 0.004 | | 0.004 | | 0.004 | |
| *Diets are balanced to contain equal levels of the antioxidant tBHQ | | | | | | |

## Appendix II – Fatty acid prolife for diets used in Chapter 3

**Fatty Acid Profile of Diets with 40 kcal% Fat (Mostly Lard) and 40 kcal% Fat (Mostly Menhaden Oil) (According to Typical Manufacturer's Analysis)**

| | 40 kcals% Fat (Mostly Lard) | 40 kcals% Fat (Mostly Menhaden) |
|---|---|---|
| | | |
| Lard | 155.5 | 0 |
| Soybean Oil | 25 | 25 |
| Menhaden Oil | 0 | 155.5 |
| | | |
| **Total (grams fat)** | **180.5** | **180.5** |
| | | |
| C12, Lauric | 0 | 0.2 |
| C14, Myristic | 1.4 | 10.7 |
| C14:1, Myristoleic | 0.8 | 0 |
| C15:0 | 0 | 0.9 |
| C16, Palmitic | 38.2 | 25.7 |
| C16:1, Palmitoleic | 5.9 | 15.1 |
| C16:2 | 0 | 2.5 |
| C16:3 | 0 | 2.3 |
| C16:4 | 0 | 2.4 |
| C17:0 | 0 | 0.8 |
| C18, Stearic | 21.6 | 4.9 |
| C18:1, Oleic | 70.0 | 21.0 |
| C18:2, Linoleic | 26.7 | 16.4 |
| C18:3, Linolenic | 3.5 | 4.3 |
| C18:4 | 0 | 4.8 |
| C20, Arachidic | 0 | 0.3 |
| C20:1 | 0 | 2.3 |
| C20:2 | 0 | 0.3 |
| C20:3 | 0 | 0.6 |
| C20:4, Arachidonic | 2.6 | 3.2 |
| **C20:5, Eicosapentaenoic** | **0** | **22.0** |
| C21:5 | 0 | 1.2 |
| C22, Behenic | 0 | 0.2 |
| C22:1, Erucic | 0 | 0.5 |
| C22:4, Clupanodonic | 0 | 0.4 |
| C22:5 | 0 | 4.4 |
| **C22:6, Docosaehexanoic** | **0** | **19.0** |
| C24, Lignoceric | 0 | 0.9 |
| C24:1 | 0 | 0.3 |
| | | |
| Total | 170.8 | 167.4 |
| | | |
| Saturated (g) | 61.2 | 44.4 |
| Monounsaturated (g) | 76.7 | 39.5 |
| Polyunsaturated (g) | 32.9 | 87.1 |
| | | |
| **Saturated (%)** | **35.9** | **26.5** |
| **Monounsaturated (%)** | **44.9** | **23.6** |
| **Polyunsaturated (%)** | **19.3** | **52.0** |

## Appendix III – Complete nutritional breakdown for diets used in Chapter 5

### Rodent Diets with 10 kcal% Fat or 40 kcal% Fat with (Mostly) Coconut Oil or Croda Oil (High DHA)

| | 10 kcal% Fat | | 40 kcal% Fat (Coconut) | | 40 kcal% Fat (Croda) | |
|---|---|---|---|---|---|---|
| **Product #** | **D12020501** | | **D12020502** | | **D12020503** | |
| | gm% | *kcal%* | gm% | *kcal%* | gm% | *kcal%* |
| Protein | 19.2 | *20* | 22.9 | *20* | 22.9 | *20* |
| Carbohydrate | 67.3 | *70* | 45.7 | *40* | 45.7 | *40* |
| Fat | 4.3 | *10* | 20.4 | *40* | 20.4 | *40* |
| Total | | *100.0* | | *100* | | *100* |
| kcal/gm | 3.85 | | 4.58 | | 4.58 | |
| | | | | | | |
| **Ingredient** | **gm** | ***kcal*** | **gm** | ***kcal*** | **gm** | ***kcal*** |
| Casein, 80 Mesh | 200 | *800* | 200 | *800* | 200 | *800* |
| L-Cystine | 3 | *12* | 3 | *12* | 3 | *12* |
| | | | | | | |
| Corn Starch | 550 | *2200* | 245 | *980* | 245 | *980* |
| Maltodextrin 10 | 150 | *600* | 150 | *600* | 150 | *600* |
| | | | | | | |
| Cellulose, BW200 | 50 | *0* | 50 | *0* | 50 | *0* |
| | | | | | | |
| Soybean Oil | 10 | *90* | 10 | *90* | 10 | *90* |
| Lard | 35 | *315* | 0 | *0* | 0 | *0* |
| Coconut Oil | 0 | | 170.5 | *1535* | 0 | *0* |
| **Croda Oil, High DHA** | 0 | *0* | 0 | *0* | 170.5 | *1535* |
| | | | | | | |
| Mineral Mix, S10026 | 10 | *0* | 10 | *0* | 10 | *0* |
| DiCalcium Phosphate | 13 | *0* | 13 | *0* | 13 | *0* |
| Calcium Carbonate | 5.5 | *0* | 5.5 | *0* | 5.5 | *0* |
| Potassium Citrate, 1 H2O | 16.5 | *0* | 16.5 | *0* | 16.5 | *0* |
| | | | | | | |
| Vitamin Mix, V10001 | 10 | *40* | 10 | *40* | 10 | *40* |
| Choline Bitartrate | 2 | *0* | 2 | *0* | 2 | *0* |
| | | | | | | |
| FD&C Yellow Dye #5 | 0.05 | *0* | 0 | *0* | 0 | *0* |
| FD&C Red Dye #40 | 0 | *0* | 0.05 | *0* | 0 | *0* |
| FD&C Blue Dye #1 | 0 | *0* | 0 | *0* | 0.05 | *0* |
| | | | | | | |
| **Total** | **1055.05** | ***4057*** | **885.55** | ***4057*** | **885.55** | ***4057*** |

## Appendix IV - Fatty acid profile for diets used in Chapter 5

**Fatty Acid Profiles based on Typical Manufacturers' Analysis**

| Fat Source | D12020501 | D12020502 | D12020503 |
|---|---|---|---|
| | | | |
| Soybean Oil | 10 | 10 | 10 |
| Lard | 35 | 0 | 0 |
| Coconut Oil | 0 | 170.5 | 0 |
| Croda Incromega E1070 | 0 | 0 | 170.5 |
| | | | |
| **Total** | **45** | **180.5** | **180.5** |
| | | | |
| C2, Acetic | 0 | 0 | 0 |
| C4, Butyric | 0 | 0 | 0 |
| C6, Caproic | 0 | 1.02 | 0 |
| C8, Caprylic | 0 | 13.13 | 0 |
| C10, Capric | 0.02 | 10.06 | 0 |
| C12, Lauric | 0.03 | 81.16 | 0 |
| C14, Myristic | 0.41 | 30.70 | 0.01 |
| C14:1, Myristoleic | 0.00 | 0 | 0 |
| C15 | 0.03 | 0 | 0 |
| C16, Palmitic | 7.79 | 15.87 | 1.04 |
| C16:1, Palmitoleic | 0.49 | 0.01 | 0.01 |
| C16:2 | 0 | 0 | 0 |
| C16:3 | 0 | 0 | 0 |
| C16:4 | 0 | 0 | 0 |
| C17 | 0.14 | 0.01 | 0.01 |
| C17:1 | 0 | 0 | 0 |
| C18, Stearic | 4.10 | 18.46 | 0.39 |
| C18:1, Oleic | 13.81 | 3.66 | 2.30 |
| C18:2, Linoleic | 13.71 | 5.23 | 5.18 |
| C18:3, Linolenic | 1.20 | 0.74 | 0.74 |
| C18:4, Stearidonic | 0 | 0 | 0 |
| C20, Arachidic | 0.09 | 0.04 | 0.04 |
| C20:1 | 0.24 | 0.03 | 0.03 |
| C20:2 | 0.28 | 0 | 0 |
| C20:3, n6 | 0.05 | 0 | 0 |
| C20:3, n3 | 0 | 0 | 0 |
| C20:4, Arachidonic | 0.10 | 0 | 0 |
| C20:4, n3 | 0 | 0 | 0 |
| **C20:5, Eicosapentaenoic** | 0 | 0 | **17.05** |
| C21:5 | 0 | 0 | 0 |
| C22, Behenic | 0.03 | 0.03 | 0.03 |
| C22:1, Erucic | 0 | 0 | 0 |
| C22:4, Clupanodonic | 0 | 0 | 0 |
| C22:5, Docosapentaenoic | 0.03 | 0 | 0 |
| **C22:6, Docosahexaenoic** | 0 | 0 | **126.17** |
| C24, Lignoceric | 0.02 | 0.02 | 0.02 |
| C24:1 | 0 | 0 | 0 |
| | | | |
| Total | 42.56 | 180.14 | 152.98 |
| | | | |
| Saturated (g) | 12.6 | 170.5 | 1.5 |
| Monounsaturated (g) | 14.5 | 3.7 | 2.3 |
| Polyunsaturated (g) | 15.4 | 6.0 | 149.1 |
| | | | |
| Saturated (%) | 29.7 | 94.6 | 1.0 |
| Monounsaturated (%) | 34.2 | 2.1 | 1.5 |
| Polyunsaturated (%) | 36.1 | 3.3 | 97.5 |
| | | | |
| n6 (gm) | 13.9 | 5.2 | 5.2 |
| n3 (gm) | 1.2 | 0.7 | 144.0 |
| Ratio (n6 to n3) | 11.2 | 7.1 | 0.04 |

## Appendix V – Complete nutritional breakdown for CRM diet used in Chapter 6

# Rat and Mouse Breeder and Grower

*Pelleted*

| NUTRIENTS | | Total | Supp (9) |
|---|---|---|---|
| **Proximate Analysis** | | | |
| Moisture (1) | % | 10.00 | |
| Crude Oil | % | 3.36 | |
| Crude Protein | % | 18.35 | |
| Crude Fibre | % | 4.23 | |
| Ash | % | 6.27 | |
| Nitrogen Free Extract | % | 57.39 | |
| **Digestibility Co-Efficients (7)** | | | |
| Digestible Crude Oil | % | 3.05 | |
| Digestible Crude Protein | % | 16.44 | |
| **Carbohydrates, Fibre and Non Starch Polysaccharides (NSP)** | | | |
| Total Dietary Fibre | % | 15.06 | |
| Pectin | % | 1.40 | |
| Hemicellulose | % | 8.85 | |
| Cellulose | % | 3.89 | |
| Lignin | % | 1.40 | |
| Starch | % | 42.37 | |
| Sugar | % | 3.90 | |
| **Energy (5)** | | | |
| Gross Energy | MJ/kg | 15.01 | |
| Digestible Energy (15) | MJ/kg | 12.27 | |
| Metabolisable Energy (15) | MJ/kg | 11.19 | |
| Atwater Fuel Energy (AFE) (8) | MJ/kg | 13.93 | |
| AFE from Oil | % | 9.08 | |
| AFE from Protein | % | 22.03 | |
| AFE from Carbohydrate | % | 68.90 | |
| **Fatty Acids** | | | |
| **Saturated Fatty Acids** | | | |
| C12:0 Lauric | % | 0.03 | |
| C14:0 Myristic | % | 0.14 | |
| C16:0 Palmitic | % | 0.33 | |
| C18:0 Stearic | % | 0.06 | |
| **Monounsaturated Fatty Acids** | | | |
| C14:1 Myristoleic | % | 0.02 | |
| C16:1 Palmitoleic | % | 0.10 | |
| C18:1 Oleic | % | 0.87 | |
| **Polyunsaturated Fatty Acids** | | | |
| C18:2(ω6) Linoleic | % | 0.96 | |
| C18:3(ω3) Linolenic | % | 0.11 | |
| C20:4(ω6) Arachidonic | % | 0.11 | |
| C22:5(ω3) Clupanodonic | % | | |
| **Amino Acids** | | | |
| Arginine | % | 1.19 | |
| Lysine (6) | % | 1.04 | 0.17 |
| Methionine | % | 0.28 | 0.02 |
| Cystine | % | 0.29 | |
| Tryptophan | % | 0.22 | |
| Histidine | % | 0.46 | |
| Threonine | % | 0.69 | |
| Isoleucine | % | 0.77 | |
| Leucine | % | 1.46 | |
| Phenylalanine | % | 0.96 | |
| Valine | % | 0.91 | |
| Tyrosine | % | 0.69 | |
| Taurine | % | | |
| Glycine | % | 1.55 | |
| Aspartic Acid | % | 1.00 | |

| NUTRIENTS | | Total | Supp (9) |
|---|---|---|---|
| Glutamic Acid | % | 3.72 | |
| Proline | % | 1.34 | |
| Serine | % | 0.78 | |
| Hydroxyproline | % | | |
| Hydroxylysine | % | | |
| Alanine | % | 0.21 | |
| **Macro Minerals** | | | |
| Calcium | % | 0.83 | 0.72 |
| Total Phosphorus | % | 0.64 | 0.19 |
| Phytate Phosphorus | % | 0.23 | |
| Available Phosphorus | % | 0.41 | 0.19 |
| Sodium | % | 0.27 | 0.22 |
| Chloride | % | 0.40 | 0.35 |
| Potassium | % | 0.69 | |
| Magnesium | % | 0.22 | 0.01 |
| **Micro Minerals** | | | |
| Iron | mg/kg | 130.65 | 60.21 |
| Copper | mg/kg | 16.42 | 6.90 |
| Manganese | mg/kg | 91.05 | 44.90 |
| Zinc | mg/kg | 86.59 | 52.86 |
| Cobalt | µg/kg | 494.92 | 420.30 |
| Iodine | µg/kg | 390.43 | 310.17 |
| Selenium | µg/kg | 265.49 | 100.34 |
| Fluorine | mg/kg | 9.63 | |
| **Vitamins** | | | |
| β-Carotene (2) | mg/kg | 1.28 | |
| Retinol (2) | µg/kg | 5218.35 | 4500.38 |
| Vitamin A (2) | iu/kg | 17376.38 | 15001.26 |
| Cholecalciferol (3) | µg/kg | 76.94 | 75.00 |
| Vitamin D (3) | iu/kg | 3077.42 | 3000.00 |
| α-Tocopherol (4) | mg/kg | 93.03 | 72.81 |
| Vitamin E (4) | iu/kg | 102.81 | 80.09 |
| Vitamin $B_1$ (Thiamine) | mg/kg | 15.84 | 9.83 |
| Vitamin $B_2$ (Riboflavin) | mg/kg | 13.28 | 11.76 |
| Vitamin $B_6$ (Pyridoxine) | mg/kg | 17.65 | 13.74 |
| Vitamin $B_{12}$ (Cyanocobalamine) | µg/kg | 78.17 | 75.00 |
| Vitamin C (Ascorbic Acid) | mg/kg | 1.80 | |
| Vitamin K (Menadione) | mg/kg | 185.05 | 180.00 |

1 mg all-rac-α-tocopherol acetate = 1.0 iu Vitamin E activity

5. 1 MJ = 239.23 Kcalories = 239.23 Calories = 239,230 calories
6. These nutrients coming from natural raw materials such as cereals may have low availabilities due to the interactions with other compounds.
7. Based on in-vitro digestibility analysis.
8. AF Energy = Atwater Fuel Energy = ((CO%/100)*9000)+ ((CP%/100)*4000)+((NFE%/100)*4000)/239.23
9. Supplemented nutrients from manufactured and mined sources.
15. Calculated.

## Appendix VI – Published abstract for work described in Chapter 3

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### Effects of dietary fatty acid intake on meal pattern

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**Effects of dietary fatty acid intake on meal pattern.** M.A. YON, L.C. PICKAVANCE. *Department of Obesity & Endocrinology, Institute of Ageing & Chronic Disease, Faculty of Health & Life Sciences, University of Liverpool, Brownlow Hill/Crown St., Liverpool L69 7ZJ, UK.* m.yon@liv.ac.uk

In humans and rodent models, obesity is associated with chronic consumption of diets high in saturated fatty acids (SFAs) and with abnormal meal patterns (MPs). In contrast, polyunsaturated fatty acids (PUFAs) attenuate weight gain, and human MP analysis reveals that they are more satiating than SFAs, when consumed as supplements in the short-term. However, the meal pattern signature of chronic PUFA consumption has never been determined. We hypothesised that it would differ from that of SFAs. For 2 months, male Wistar rats were fed isocaloric high-fat diets, providing 40% kcal as fat, either from fish oil (PUFA) or lard (SFA; $n = 8$/group). Body weight was monitored weekly, and plasma hormones and metabolites involved in energy metabolism were measured fortnightly by ELISA. Terminal fat pads were also weighed. MPs were recorded by automated cages (TSE Systems, Germany). The groups showed comparable weight gain ($p > 0.05$), supported by equivalent food intake and circulating levels of glucose, leptin and insulin (all $p > 0.05$). However, there was a trend towards greater perirenal fat pad mass in SFA-fed rats (+22%; $p = 0.06$) and 50% lower triglyceride levels in PUFA-fed rats ($p = 0.002$), consistent with known effects on adiposity and metabolism. Overall, MPs were also similar between the groups ($p > 0.05$): despite reductions in nocturnal intake, and number of meals, within-meal consumption rate increased, while meal duration was unchanged. Diurnal exceeded nocturnal satiety in both groups (+98%; $p = 0.005$), and was marginally enhanced throughout by the PUFA diet, though not significantly (+22%; $p > 0.05$). Therefore, we have shown for the first time that meal pattern signatures are similar for calorically matched, chronic high-PUFA and -SFA consumption.

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